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Signal transduction of plant organ senescence and cell death

Edited by
Ying Miao, Hongwei Guo, Ralf Oelmüller and Zhonghai Li

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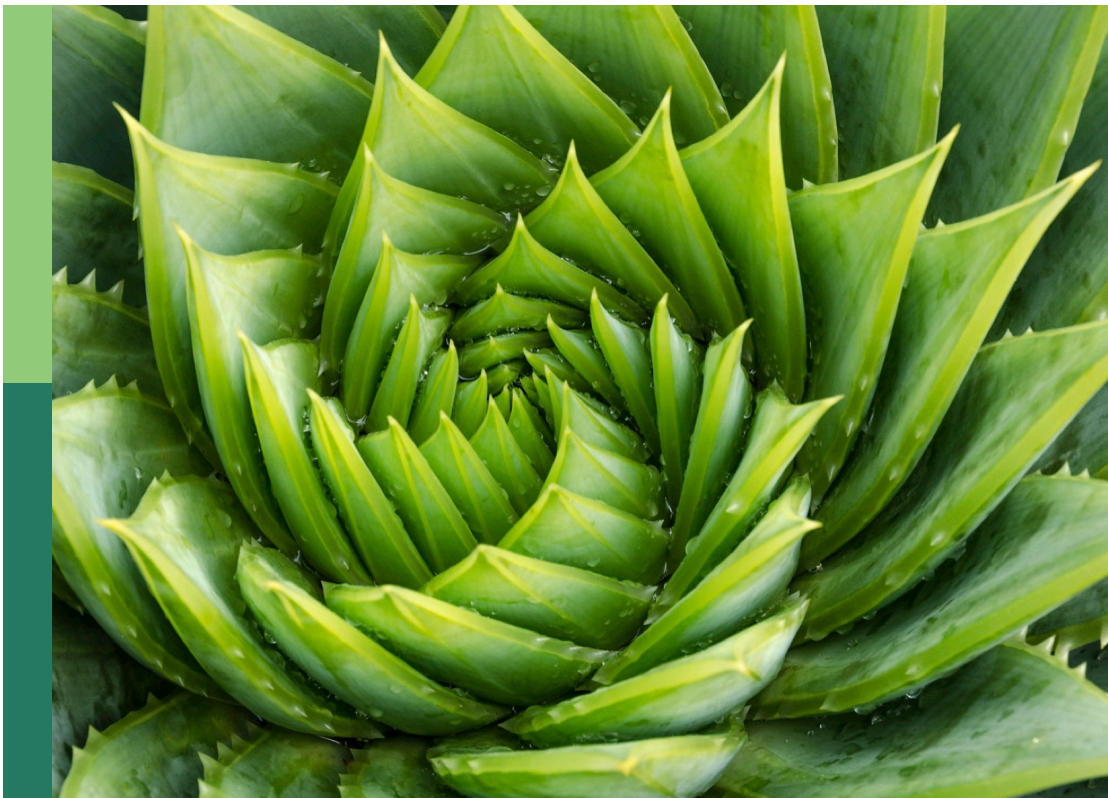

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Signal transduction of plant organ senescence and cell death

Topic editors

Ying Miao — Fujian Agriculture and Forestry University, China
Hongwei Guo — Peking University, China
Ralf Oelmüller — Friedrich Schiller University Jena, Germany
Zhonghai Li — Beijing Forestry University, China

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Table of contents

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Anna N Stepanova,
North Carolina State University,
United States

*CORRESPONDENCE
Ying Miao
ymiao@fafu.edu.cn

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Editorial: Signal transduction of plant organ senescence and cell death

Zhonghai Li[1], Ralf Oelmüller[2], Hongwei Guo[3] and Ying Miao[4]*

[1]State Key Laboratory of Tree Genetics and Breeding, College of Biological Sciences and Technology, Beijing Forestry University, Beijing, China, [2]Matthias Schleiden Institute, Plant Physiology, Friedrich-Schiller-University Jena, Jena, Germany, [3]Key Laboratory of Molecular Design for Plant Cell Factory of Guangdong Higher Education Institutes, Department of Biology, Southern University of Science and Technology (SUSTech), Shenzhen, Guangdong, China, [4]Fujian Provincial Key Laboratory of Plant Functional Biology, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China

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Editorial on the Research Topic
Signal transduction of plant organ senescence and cell death

The senescence of plant organs, the final stage of organ development, is a form of programmed cell death (Miao and Zentgraf, 2007). It is characterized by the functional transition from nutrient assimilation to nutrient remobilization, which is crucial for plant fitness and affects crop yield, quality, and horticultural performance (Guo and Gan, 2005; Lim et al., 2007). Although it has been reported that leaf senescence impacts photosynthesis, nutrient mobilization, stress responses, and productivity (Guo et al., 2021), the contributions of a myriad of natural parameters, such as organ age, coordination between different regulatory pathways, source-sink relationships, nutrient remobilization, and anterograde/retrograde signal transduction during organ senescence, remain to be unraveled.

This Research Topic compiles a total of ten articles, four reviews, and six research studies, covering five topics: i) New discoveries in the multiple layers of regulation of leaf senescence; ii) Recent progress in the regulation of leaf senescence by classical and peptide hormones; iii) Novel signaling components regulating organ senescence; iv) New mechanisms for nutrient deficiency-induced leaf senescence; and v) Latest breakthroughs in leaf senescence research methods and techniques.

Leaf senescence is a systematic physiological process that involves several tiers of regulation, including at the level of chromatin remodeling, as well as at the transcriptional, translational, and post-translational levels, as revealed by multi-omics analyses. (Woo et al., 2013; Woo et al., 2019) Miao's team reported that histone acetylation (H3K9ac) enrichment accompanied the transcriptional induction of senescence-associated genes (SAGs) during leaf senescence in Arabidopsis and rice (Huang et al., 2018; Zhang et al.,

2022a; Huang et al., 2022). In Arabidopsis, histone deacetylase 15 (HDA15) interacting with an ssDNA-binding protein WHIRLY1 acts as a repressor of downstream gene transcription, leaf senescence, and flowering (Huang et al., 2022). Zareen et al. reported that histone deacetylase 9 (HDA9) and POWERDRESS (PWR) complex recruiting a transcriptional corepressor HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE15 (HOS15) acts as a positive regulator to promote leaf senescence upon aging and dark stress by repressing the histone acetylation at several gene loci, such as *NPX1*, *APG9*, and *WRKY57*. However, many unidentified mechanisms at the epigenetic modification level still need to be clarified.

Long non-coding RNAs (lncRNAs) are a type of non-coding RNAs that are emerging as hidden players in many biological processes (Statello et al., 2021). In this Research Topic, Kim et al. performed comprehensive analyses of RNA-seq data representing all leaf developmental stages to determine the genome-wide lncRNA landscape during Arabidopsis leaf aging, providing a valuable resource of age-related (AR) lncRNAs and proposing a potential gene regulatory network of leaf senescence that links the function of protein-coding mRNAs and AR-lncRNAs. Recent advances in the fields of non-coding RNAs, epigenetic modifications, and alternative splicing in the regulation of leaf senescence have all been reviewed (Guo et al., 2021; Zhang et al., 2021; Miryeganeh, 2022).

Protein phosphorylation/dephosphorylation plays a crucial role in the leaf senescence process. Controlled protein dephosphorylation by protein phosphatases is vital to containing the extent of senescence. Several protein phosphatases that positively or negatively influence the induction or progression of this process have been identified (Zhang and Gan, 2012; Xiao et al., 2015; Durian et al., 2020). Protein kinase, in turn, functions in signal transduction *via* the phosphorylation of downstream signaling components to activate the regulatory network (Ahmad and Guo, 2019). Miao et al. reported that MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) KINASE KINASE1, MEKK1, can take a shortcut and directly phosphorate the WRKY53 protein as well as activate *WRKY53* gene expression and leaf senescence (Miao et al., 2007). Wu et al. show that MPK3 and MPK6, two Arabidopsis MAPKs, and their two upstream MAPK kinases, MKK4 and MKK5, act *via* the MKK4/5, MPK3/6, and MATRIX METALLOPROTEINASE (MMP) At2/At3 cascade as key regulators of leaf senescence. Yang et al. reviewed the recent progress in plant leaf senescence-related kinases and summarized the current understanding of the function of kinases in senescence signal perception and transduction.

The role of classic phytohormones, such as abscisic acid (ABA), ethylene, jasmonic acid (JA), salicylic acid (SA), brassinolide (BR), gibberellin (GA), and auxin indole-3-acetic acid (IAA) that function as important signaling molecules in plants and contribute to the onset and progression of leaf senescence, has been well documented (Guo et al., 2021). Increasing evidence now shows that peptide hormones CLAVATA3/ESR-RELATED (CLEs), Phytosulfokine (PSK), and INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) or IDA-like (IDLs) peptides are also involved in the regulation of leaf senescence, expanding the repertoire of signaling molecules that control leaf senescence (Zhang et al., 2022b; Zhang et al., 2022c). In this issue, Guo et al. reported that the IDL6 peptide is a positive regulator of leaf senescence. Huang et al. presented recent advances in our understanding of leaf senescence regulation by classical and peptide hormones.

Organ senescence, a type of programmed cell death, leads to the massive retrieval of nutrients from senescing organs to the rest of the plant (Rogers, 2013; Schippers et al., 2015). In addition to carbohydrate and energy remobilization during leaf, petal, and seed senescence (Chrobok et al., 2016; Huang et al., 2020; Zhang et al., 2021; Zhu et al., 2022), many macronutrients, such as magnesium (Mg), iron (Fe), and nitrogen (N), also get recycled and channeled into essential cellular processes such as an extensive range of metabolic, regulatory, and structural activities (Guo et al., 2015; Guo et al., 2021). The deficiency or excess of these nutrients seriously affects plant growth and development (Shi et al., 2012; Tanoi and Kobayashi, 2015; Yang and Udvardi, 2018). For example, Mg as a constituent of magnesium porphyrin plays a role in retrograde signaling and ABA-induced senescence (Koussevitzky et al., 2007). In this issue, Kocourkova et al. showed that in *PHOSPHOLIPASE Dα1*-deficient mutant plants, *pldα1-1*, higher accumulation of ABA and JA, and impaired homeostasis of Mg, potassium, and phosphate were observed under high-Mg^{2+} conditions. Furthermore, high Mg^{2+} also led to an increase in starch and proline content in Arabidopsis plants. PLDα1 was concluded to act as a negative regulator of high-Mg^{2+}-induced leaf senescence. Finally, in this article collection, Sakuraba et al. reviewed the current understanding of the molecular mechanisms associated with N starvation-induced leaf senescence.

Given that senescence is affected by numerous developmental and environmental signals, such as biotic and abiotic stresses (Guo et al., 2021), research on organ senescence requires systematic approaches and sophisticated experimental designs. Plant scientists are searching for rapid experimental systems to reveal the molecular regulatory mechanisms of leaf senescence controlled by multiple factors. Protoplasts are an effective experimental system for employing rapid and systematic cellular approaches to dissect gene function in Arabidopsis (Tyurin et al., 2020) and to perform genetic manipulation in crops (Ghogare et al., 2021). In this issue, Kim et al. established a transient gene expression assay in *Arabidopsis* protoplasts and validated this system by monitoring the differential expression of *LUCIFERASE*-based reporters driven by the promoters of SAGs (*SEN4-LUC* and *SAG12-LUC*) (Doan et al., 2022). This approach provides a valuable system for studying senescence at the cellular and molecular levels in various species.

This article collection is a testament to the notion that, even though impressive progress has been made in the identification and functional analysis of a large number of SAGs in plants, many urgent scientific questions remain in this field, such as when plant senescence is initiated or how senescence signals are transmitted between organelles, cells, tissues, and organs, as well as how to best address the molecular mechanisms underlying cell senescence.

With the application of single-cell multi-omics analysis and gene-editing technologies, such as CRISPR/Cas9, the precise mechanisms governing cell senescence will be deciphered, and a wide variety of genome-modified stay-green crops will be developed and commercialized in the foreseeable future.

Author contributions

ZL and YM prepared drafts of the manuscript, and RO and HG revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Phospholipase Dα1 Acts as a Negative Regulator of High Mg^{2+}-Induced Leaf Senescence in Arabidopsis

*Daniela Kocourková, Kristýna Kroumanová, Tereza Podmanická, Michal Daněk and Jan Martinec**

Institute of Experimental Botany of the Czech Academy of Sciences, Prague, Czechia

Magnesium (Mg^{2+}) is a macronutrient involved in essential cellular processes. Its deficiency or excess is a stress factor for plants, seriously affecting their growth and development and therefore, its accurate regulation is essential. Recently, we discovered that phospholipase Dα1 (PLDα1) activity is vital in the stress response to high-magnesium conditions in Arabidopsis roots. This study shows that PLDα1 acts as a negative regulator of high-Mg^{2+}-induced leaf senescence in Arabidopsis. The level of phosphatidic acid produced by PLDα1 and the amount of PLDα1 in the leaves increase in plants treated with high Mg^{2+}. A knockout mutant of PLDα1 (*pldα1-1*), exhibits premature leaf senescence under high-Mg^{2+} conditions. In *pldα1-1* plants, higher accumulation of abscisic and jasmonic acid (JA) and impaired magnesium, potassium and phosphate homeostasis were observed under high-Mg^{2+} conditions. High Mg^{2+} also led to an increase of starch and proline content in Arabidopsis plants. While the starch content was higher in *pldα1-1* plants, proline content was significantly lower in *pldα1-1* compared with wild type plants. Our results show that PLDα1 is essential for Arabidopsis plants to cope with the pleiotropic effects of high-Mg^{2+} stress and delay the leaf senescence.

Keywords: ***Arabidopsis thaliana*, magnesium homeostasis, phospholipase D, leaf senescence, starch, proline, abscisic acid, jasmonic acid**

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Edited by:
Zhonghai Li,
Beijing Forestry University, China

Reviewed by:
Mohammad Israil Ansari,
University of Lucknow, India
Peipei Xu,
Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (CAS), China

****Correspondence:***
Jan Martinec
martinec@ueb.cas.cz

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INTRODUCTION

Magnesium (Mg^{2+}) is a macronutrient involved in essential cellular processes such as photosynthesis, nucleic acid and protein synthesis, energy metabolism, etc. (Guo et al., 2016). Its deficiency or, on the contrary, its excess is a stress factor for plants, seriously affecting plant growth and development. Therefore, accurate regulation of intracellular magnesium level is essential. The knowledge of the mechanisms activated in Mg^{2+} deficiency is relatively good. The mechanisms associated with the regulation of cellular Mg^{2+} under high-Mg^{2+} conditions are less known. High concentrations of Mg^{2+} together with low concentrations of Ca^{2+} occur, for example, in serpentine soils. Recently, high-magnesium water and soils have been considered as an emerging environmental and food security issues (Qadir et al., 2018). For non-adapted plants, high-Mg^{2+} conditions are strongly inhibitory to growth.

Magnesium is absorbed by plants in its ionic form from the soil. Plants growing in soils with high Mg^{2+} can mitigate Mg^{2+} toxicity by limiting internal Mg^{2+} accumulation and/or Mg^{2+} excretion from leaves. Sequestration of additional Mg^{2+} into the vacuole under high Mg^{2+} conditions appears to play a central role in tolerance to high Mg^{2+} (Hermans et al., 2013). The involvement of a network of calcineurin B-like calcium sensor proteins (CBL) CBL2/3, CBL-interacting protein kinases (CIPK) CIPK3/9/23/26, and sucrose non-fermenting-1-related protein kinase2 (SnRK2) SRK2D/E/I in the high-Mg^{2+} response of Arabidopsis has been shown (Mogami et al., 2015; Tang et al., 2015; Chen et al., 2018). Based on the altered sensitivity of the corresponding knock-out mutants to high-Mg^{2+} conditions, several other proteins were identified as participants in the high-Mg^{2+} response. Vacuolar-type H^+-pyrophosphatase (AVP1; Yang et al., 2018), magnesium transporter 6 (MGT6; Yan et al., 2018), and *mid1*-complementing activity 1, 2 (MCA1/2; Yamanaka et al., 2010) are required for tolerance to high Mg^{2+} because their knock-out mutants were hypersensitive to high-Mg^{2+} conditions. In contrast, knock-out mutants of cation exchanger 1 (CAX1; Cheng et al., 2003; Bradshaw, 2005) and nucleoredoxin 1 (NRX1; Niu et al., 2018) were more resistant to high Mg^{2+}. Interestingly, MCA1/2, CAX1, and NRX1 are involved in the regulation of cytosolic Ca^{2+} concentration, suggesting a link between calcium homeostasis and high Mg^{2+} tolerance. In addition, the involvement of ABA signaling in response to high magnesium conditions has been demonstrated. An increase in ABA content and expression of ABA biosynthetic genes was reported under high magnesium conditions (Visscher et al., 2010; Guo et al., 2014). Moreover, the ABA – insensitive mutant *abi1-1* was less sensitive to high magnesium treatment than WT (Guo et al., 2014).

Recently, we discovered that phospholipase Dα1 (PLDα1) activity is vital in the stress response to high-magnesium conditions in Arabidopsis. The T-DNA insertion mutant *pldα1* was hypersensitive to elevated magnesium levels and showed reduced primary root length and fresh weight. PLDα1 activity increases rapidly following high-Mg^{2+} treatment. Moreover, high-Mg^{2+} treatment was shown to disrupt K^+ homeostasis.

Plant phospholipases D (PLD) cleave common phospholipids such as phosphatidylcholine releasing phosphatidic acid (PA) and free head group. PLDα1, the most abundant PLD member in Arabidopsis, has been reported to play a role in stress responses such as plant-microbe interaction, wounding, freezing, dehydration, and salinity (Wang et al., 2014; Ruelland et al., 2015; Hong et al., 2016). The PA apparently serves as a key signaling molecule in the above responses (Pokotylo et al., 2018).

Leaf senescence is a normal manifestation of plant ageing and represents the final stage of its development. There is also senescence induced by environmental stresses such as drought, cold, heat, low light, and pathogen attack (Zhang and Zhou, 2013; Sade et al., 2018), nutrient deficiencies such as nitrogen (Meng et al., 2016), potassium (Cao et al., 2006; Li et al., 2012; Wang et al., 2012), or magnesium (Tanoi and Kobayashi, 2015). Not only deficiency but also excess of nutrients leads to premature senescence of leaves. Exposure of sunflower plants to elevated K^+ concentration resulted in premature leaf senescence (Santos, 2001). Ionic imbalance caused by salt stress also causes premature leaf senescence. The regulatory role of ROS (Allu et al., 2014) and transcription factor ANAC092 (Balazadeh et al., 2010) was revealed here. Interestingly, both ROS and ANAC092 are also involved in the regulation of developmental senescence. Thus, there is an overlap between stress-induced senescence and developmental senescence. In addition to ROS and specific transcription factors, the phytohormones jasmonic acid, ABA and cytokinins (CK) also play important roles in senescence processes.

This study shows that high external magnesium concentration triggers leaf senescence in Arabidopsis. Moreover, the knockout mutant of PLDα1 exhibits premature leaf senescence under high-Mg^{2+} conditions compared with WT. Under high-Mg^{2+} conditions, we also observed impaired ion homeostasis of *pldα1*. Furthermore, hormone, starch, and proline accumulation were altered in *pldα1* plants senescing under high Mg^{2+}. From these results, we conclude that PLDα1 functions as a negative regulator of high-Mg^{2+} induced leaf senescence.

MATERIALS AND METHODS

Plant Materials

Arabidopsis thaliana Col-0 was used in the study. Knockout line *pldα1-1* (SALK_067533) was obtained from the NASC. Complemented lines *pldα1-1*Com1 and *pldα1-1*Com2 were described previously (Kocourková et al., 2020).

Plant Cultivation

Phenotypic experiments were performed on agar plates and in hydroponics. On vertical agar plates, plants were grown for 10 days on ½ MS, 1% agar (Sigma) and then transplanted into either control plates [½ MS (Duchefa), 1% agar] or high-Mg^{2+} plates [½ MS, 15 mM $MgCl_2$, 1% agar (Sigma)] and grown for another 7 days. Plates were kept in a growth chamber at 22°C during the day, 21°C at night, under long day (16 h of light) conditions at 100 µmol m^{-2} s^{-1} of light. Hydroponic plant cultivation was described in Kocourková et al. (2020). Twenty-four-day-old hydroponically cultivated plants were treated with either ½ Hoagland's solution (control) or ½ Hoagland's solution with 15 mM $MgSO_4$ added. The plants were grown in a growth chamber at 22°C during the day (light intensity of 100 µmol m^{-2} s^{-1}) and 21°C at night in a 10-h day/14-h night mode.

PLDα1 Activity

To determine *in vitro* activity, extracts from a mixed leaf sample from the 3rd, 4th, 5th, and 6th oldest true leaves of the plant (=mixed leaf sample) were prepared from hydroponically grown plants treated with 0 or 15 mM $MgSO_4$ for 1, 2, 3, 4, 7, and 10 days. The leaves were frozen in liquid nitrogen. Samples were homogenized and buffer (per 1 mg sample 5 µl buffer) consisting of 0.4 M sucrose, 0.1 M $MgCl_2$, 0.1 M KCl, 50 mM HEPES-NaOH pH 7.5, Complete protease inhibitor coctail (Roche) and Pierce Phosphatase Inhibitor Mini Tablets (Thermo

Fisher Scientific) was added to the homogenized samples. The samples were centrifuged for 10 min at 6,010 *g* at 4°C. The supernatant was transferred into a new tube and the samples were centrifuged for 90 min at 27,400 *g* at 4°C. The supernatant was collected and the protein concentration was measured using a Coomassie Plus Protein Assay (Thermo Scientific).

The enzymatic reaction 100 µl contained 15 µl of sample (1 µg/µl), 50 mM MES (pH 6.5, NaOH), 20 mM $CaCl_2$ and 25 µl of substrate solution. The substrate solution contained 4 µM fluorescent PC (BODIPY-PC, Invitrogen™ by Thermo Fisher Scientific), 25 µM 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids), 0.015% sodium deoxycholate and 50 mM MES buffer (pH 6.5). The substrate solution was incubated at room temperature for 30 min and then sonicated for 10 min. The reaction was started by adding the substrate and run for 30 min at 25°C with shaking at 500 rpm. Lipids were extracted according to Krckova et al. (2018). The lipids were separated first by the mobile phase methanol/chloroform/water/acetic acid (21/15/4/0.8) and after drying by the mobile phase chloroform/methanol/water (26/9/1). The plates were laser-scanned using Sapphire™ Biomolecular Imager (Azzure Biosystems) and evaluated using Azure Spot 2.2 software. The phosphatidic acid standard was prepared using commercial phospholipase D (Sigma Aldrich; Pejchar et al., 2010).

Western Blot Analysis

Western blot analysis was performed as described previously (Kocourková et al., 2020) with minor changes. Protein extracts were prepared as described above for TLC analysis. Proteins were separated on 10% SDS PAGE and transferred by wet blot overnight on a nitrocellulose membrane. PLDα1 protein was detected with anti-PLDα1/2 antibody (Agrisera) diluted 1: 2000 in 3% low fat milk in TBS-T. Goat anti-rabbit (Bethyl) in 5% low fat milk were used as a secondary antibody. Precision plus protein dual color standard (Biorad) was used and the position of the bands after blot transfer was marked on the membrane with a Western blot marker pen (Abcam). To control protein transfer, the membrane was stained with Novex reversible membrane protein stain (Invitrogen) according to the manufacturer's instructions.

Chlorophyll Content

Samples were frozen in liquid nitrogen and homogenized. Chlorophyll was extracted into ethanol. Samples with ethanol were heated to 65°C, left overnight at 4°C and centrifuged (10,000 *g*, 10 min). The absorbance of the extracts was measured at 649 nm and at 665 nm and the chlorophyll content was calculated according to Ritchie (2006) and expressed as mg per g fresh weight.

Gene Transcription Analysis

Gene transcriptions were measured either in whole aboveground parts of plants grown on agar treated with 0 or 15 mM $MgCl_2$ for 7 days or in the mixed leave sample of plants grown hydroponically treated with 0 or 15 mM $MgSO_4$ for 3 days. Measurement of gene expression was done according to Kocourková et al. (2020) with minor changes. Briefly, RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and genomic DNA removed using a Turbo DNA-free Kit (Applied Biosystems). Transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with 0.5 µg RNA per reaction. Quantitative PCR was performed with a LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480 System (Roche). The sequences of the primers used are listed in **Supplementary Table S1**.

Ion Leakage

Rosettes of plants grown on agar and treated with 0 or 15 mM $MgCl_2$ for 7 days were immersed in deionized water. Electrolyte leakage was measured with a COND 70 portable Conductivity Meter after 1 h of incubation at room temperature. The samples were then autoclaved and the total conductivity of the extract was measured. The results were expressed as a proportion of the total conductivity in %.

Measurement of Nutrient Content

Seedlings were grown for 10 days on half-strength MS media, after which they were transferred to agar plates with 0 or 15 mM $MgCl_2$ for 7 days. Plates were kept in a growth chamber at 22°C during the day, 21°C at night, under long day (16 h of light) conditions at 100 µmol m^{-2} s^{-1}. Samples (pooled plants, ~100 mg dry weight) were digested with HNO_3: HCl (6:1, v:v) and P, Mg^{2+}, K^+, and Ca^{2+} content was determined with inductively coupled plasma optical emission spectroscopy (Spectroblue, Spectro, Germany) analysis in the laboratory of Ekolab Žamberk, Czechia.

Starch Staining

For starch staining the 10-day-old plants grown on agar plates treated for 3 days with 0 or 15 mM $MgCl_2$ and hydroponically grown 24-day-old plants treated for 3 days with 0 or 15 mM $MgSO_4$ were used. Plants were collected at the end of the dark period. Chlorophyll was removed by immersion in 80% hot ethanol. The ethanol was changed until the rosettes were completely discolored. The rosettes were washed with water and then stained for 10 min with Lugol solution (Sigma) and washed for 1 h in water at room temperature. The plants were then scanned on a Scanner Epson Perfection V800 Photo (Epson).

Phytohormone Analysis

Phytohormones were analyzed according to Prerostova et al. (2021). Briefly, samples (20–45 mg FW leaves) were homogenized and extracted with 100 µl 50% acetonitrile solution. The extracts were centrifuged at 4° C and 30,000 *g*. The supernatants were applied to SPE Oasis HLB 96-well column plates (10 mg/well; Waters, United States) and then eluted with 100 µl 50% acetonitrile. The pellets were then re-extracted. Phytohormones in each eluate were separated on Kinetex EVO C18 column (Phenomenex, United States). Hormone analysis was performed with a LC/MS system consisting of UHPLC 1290 Infinity II

coupled to 6,495 Triple Quadrupole Mass Spectrometer (Agilent, United States).

Proline Accumulation Measurement

Proline content was measured in the mixed leaf sample of plants grown hydroponically treated with 0 or 15 mM $MgSO_4$ for 3, 4, and 7 days with ninhydrin method (Bates et al., 1973). The samples were homogenized and proline was extracted into 3% sulfosalicylic acid (SSA, 5 µl/mg fresh weight). The samples were centrifuged (5 min at maximum speed) and supernatant was collected. The reaction mixture (180 µl) consisted of 30 µl sample, 96 µl glacial acetic acid, 24 µl 6 M orthophosphoric acid, 30 µl 3% SSA and 1.5 mg ninhydrin. The reaction was run for 1 h at 96°C. Then the samples were cooled on ice and 300 µl of toluene was added. The absorbance in the upper phase was measured at 520 nm.

RESULTS

PLDα1 Activity and Amount of PLDα1 Increase in Leaves of Mg^{2+} Treated Plants

We had previously reported that increased Mg^{2+} concentration rapidly induces PLDα1 activity in Arabidopsis roots (Kocourková et al., 2020). Here, we monitored PLDα activity after Mg^{2+} treatment in Arabidopsis leaves. 24-day-old hydroponically grown plants were treated with 15 mM $MgSO_4$ for 1–10 days. Leaves from control and treated plants were harvested, homogenized, and the enzyme activity of PLDα was determined *in vitro*. PLDα activity increased throughout the observation period (1–10 days) compared with the control (**Figures 1A,B**). Higher PLDα1 activity compared with the control was observed after 2 days of treatment with 15 mM $MgSO_4$ (**Figures 1A,B**) and increased 1.3-fold. The maximum activity was observed on the seventh day, when it increased almost 17-fold.

PLDs cleave common phospholipids such as phosphatidylcholine, releasing PA and the free head group, e.g., choline. PA is also the product of diacylglycerol kinase activity as well as the substrate for PA phosphatase, among other enzymes (Ruelland et al., 2015). Therefore, the PA level does not necessarily correlate with PLD activity. Moreover, there are several isoforms of PLD in Arabidopsis that differ in their biochemical properties (Kolesnikov et al., 2012). To measure PLD activity *in vitro*, we chose the optimal conditions for PLDα activity. PLDα activity was also determined in control and Mg^{2+}-treated PLDα1 knockout plants (*pldα1-1*). In *pldα1-1*, no increase in PA level (PLDα activity) was observed under either control or high Mg^{2+} conditions (**Supplementary Figure S1**). Thus, the activity of the PLDα1 isoform is responsible for the observed increase in PA level. The increase in PLDα1 activity may be due to activation of PLDα1 or a higher level of PLDα1 protein, or both. The amount of PLDα1 in the leaves of control and treated plants was examined by western blot using the anti-PLDα1,2 antibody. The results clearly show that the level of PLDα1 increases after Mg^{2+} treatment (**Figures 1C,D**). The difference between control and treated plants was detectable after 3 days of Mg^{2+} treatment.

Determination of the activity and level of PLDα1 was performed in samples consisting of mature third, fourth, fifth, and sixth leaves. Senescence symptoms were slightly visible in these leaves on the seventh day. However, the same trend, increased activity of PLDα1, was observed in the young leaves (7th–10th), which showed no visible signs of senescence (**Supplementary Figure S2**). Therefore, we hypothesize that changes in PLDα1 activity and content are not downstream of the manifestation of leaf senescence.

These results show that both PLDα1 activity and PLDα1 levels increase in Arabidopsis leaves after treatment with Mg^{2+}.

High Magnesium Induces Premature Leaf Senescence in PLDα1

We found (Kocourková et al., 2020) that 12-day-old Arabidopsis seedlings of *pldα1* under high-Mg^{2+} conditions had shorter primary and lateral roots and lower fresh weight. Here, we noticed higher yellowing or yellow spots on *pldα1-1* leaves after 15 mM $MgSO_4$ treatment of 24-day-old plants (**Figures 2A,B**). We chose 15 mM $MgSO_4$ for the experiments because no serious adverse effects were observed on the treated plants during the first 4 days of treatment. Besides higher yellowing, the fresh weight of *pldα1-1* rosettes was less than half compared with WT, and the chlorophyll content of *pldα1-1* decreased by about 35% compared with WT (**Figures 2C,D**). This indicates premature leaf senescence of *pldα1-1* plants.

To further characterize the observed phenomenon, we additionally monitored high-Mg^{2+}-induced senescence by determining the expression of senescence genes and measuring ion leakage as a marker of membrane damage. To verify that the observed premature senescence was exclusively related to PLDα1, we also included two *pldα1-1*complemented (*pldα1-1*Com1 and Com2) lines (Kocourková et al., 2020). As in the adult plants, the fresh weight of *pldα1-1* rosettes was significantly lower (36%) compared with the WT and complemented lines (**Figures 2E,F**). Expressions of Senescence-Associated Genes 13 (SAG13, At2g29350) and the transcription factor ANAC092/NAC2/ORE1 (At5g39610) are commonly used as markers of senescence (Balazadeh et al., 2010; Bresson et al., 2018). Expressions of these genes were higher in all Mg^{2+}-treated WT, *pldα1-1* and complemented plants in comparison with untreated controls. Hence, high-Mg^{2+} induced transcriptional changes accompanying leaf senescence in all studied genotypes. Moreover, in *pldα1-1* plants, *SAG13* and *ANAC092* expression was notably higher than in WT or complemented lines. Expression of *SAG13* was approximately 2,000-fold higher in *pldα1-1* seedlings treated with high Mg^{2+} than in the untreated control, whereas for WT the increase was only 314-fold higher than in the untreated control (**Figure 2G**). Similarly, the expression of *ANAC092* was increased 12-fold in *pldα1-1*, whereas it increased only 2-fold in WT (**Figure 2H**). Membrane damage was estimated by measuring ion leakage. After Mg^{2+} treatment, ion leakage reached 7.7% in *pldα1-1*, while it was only 3% in WT (**Figure 2I**).

These results demonstrate that high-Mg^{2+} treatment induces premature leaf senescence and that *pldα1-1* plants reveal

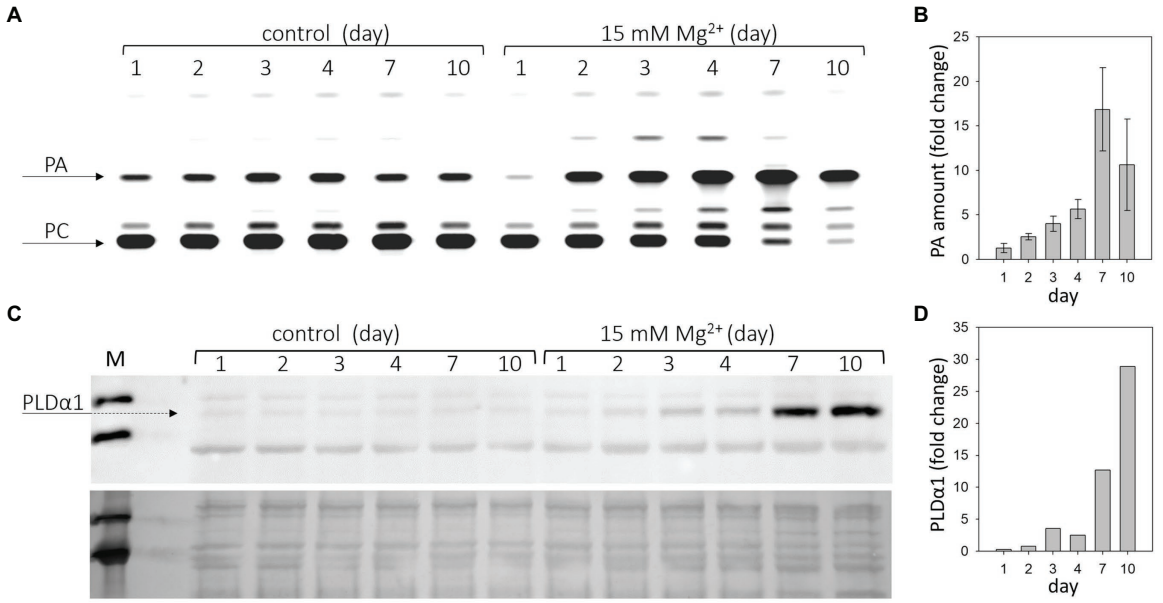

FIGURE 1 | Phospholipase Dα1 (PLDα1) activity and amount increases in response to high Mg^{2+} stress. Twenty-four-day-old hydroponically grown plants were treated with 15 mM $MgSO_4$ and sampled after 1, 2, 3, 4, 7, and 10 days after the treatment. **(A)** Thin layer plate showing phosphatidic acid, product of PLDα1 activity. PLDα1 activity was measured in leave extracts from leaves 3–6 from plants treated with $MgSO_4$. **(B)** Relative increase of PA with $MgSO_4$ treatment over time. Values represent mean ± SE, $n = 3$ biological experiments. **(C)** Western blot detection of PLDα1 in protein extracts from leaves. Each lane was run with 15 μg of protein, upper panel – western blot, lower panel – loading control – membrane stained with Novex reversible membrane protein stain. **(D)** Quantification of PLDα1 protein. The experiments were repeated three times with similar results. PA, phosphatidic acid; PC, phosphatidylcholine; and M, molecular marker.

significantly higher premature leaf senescence in comparison to WT. Premature senescence after high-Mg^{2+} treatment was observed in both 10-day-old seedling and 3-week-old mature plants. Thus, we hypothesize that PLDα1 acts as negative regulator of high-Mg^{2+} induced leaf senescence.

Levels of Plant Hormones Are Altered in High-Mg^{2+} Conditions

Plant hormones are one of the key components involved in the processes of leaf senescence, influencing all stages, initiation, progression and terminal phase, of leaf senescence (Lim et al., 2007). Additionally, Guo et al. (2014) reported increase level of abscisic acid (ABA) in response of Arabidopsis Landsberg erecta to high-Mg^{2+} conditions. Hence, we measured range of phytohormones in WT and *pldα1-1* in control and high-Mg^{2+} conditions after 2 days of high-Mg^{2+} treatment (**Supplementary Table S2**). Principal component analysis of all measured shoot phytohormones showed a clear separation on the PC1 axis of both control and high-Mg^{2+} conditions and genotypes (WT vs. *pldα1-1*). There was also a separation on the PC2 axis between WT and *pldα1-1* genotype in high-Mg^{2+} conditions (**Figure 3A**). These results demonstrate robust hormonal response to high-Mg^{2+} conditions in WT as well as involvement of PLDα1 in this hormonal response.

In WT, the highly active cytokinin (CK) trans-zeatin (tZ) and its riboside (tZR) lowered after Mg^{2+} treatment. Also, the content of the precursor trans-zeatin riboside monophosphate (tZRMP) lowered in high Mg^{2+} treated shoots. On the opposite, the levels of the stress-related CKs cis-zeatin (cZ), its riboside (cZR), and phosphate (cZRMP) increased after high Mg^{2+} treatment in WT.

The high-Mg^{2+} treatment up-regulated the production of auxin indole-3-acetic acid (IAA) in WT plants. The level of IAA precursor, indole-3-acetamide (IAM) increased under the same conditions as well. Also, deactivation of production of IAA irreversible amino acid conjugate, IAA-glutamate significantly decreased after high Mg^{2+} treatment in WT.

ABA and its catabolites phaseic acid and 9-hydroxy-abscisic acid (9OH-ABA) elevated about three times in WT shoot of high-Mg^{2+} treated plants in comparison with non-treated plants.

Jasmonic acid (JA) was greatly up-regulated (about 17 times) in high-Mg^{2+} conditions. Similarly, levels of JA precursor, cis-12-oxo-phytodienoic acid (cisOPDA) and JA metabolites, jasmonic acid methyl ester (JA-Me) and dinor-12-oxo-phytodienoic acid (dinorOPDA) significantly increased. Great increase of shoot salicylic acid (SA) level was detected in high-Mg^{2+} treated WT plants as well.

Under control conditions, no significant differences were found between WT and *pldα1-1* in the levels of all hormones measured, except for tZR. Under high-Mg^{2+} conditions, changes of some of the hormones differed between WT and *pldα1-1* (**Figure 3B**; **Supplementary Table S2**). Increase of SA was the same in WT and *pldα1-1*. Also, the increase of IAA was the same in WT and *pldα1-1*. However, higher increase was observed in the levels of both IAA precursor, IAM and IAA metabolite oxo-IAA-glucose ester (OxIAA-GE). Increase of JA (but not its precursor or metabolites) was significantly higher in *pldα1-1* in comparison with WT (**Figure 3A**;

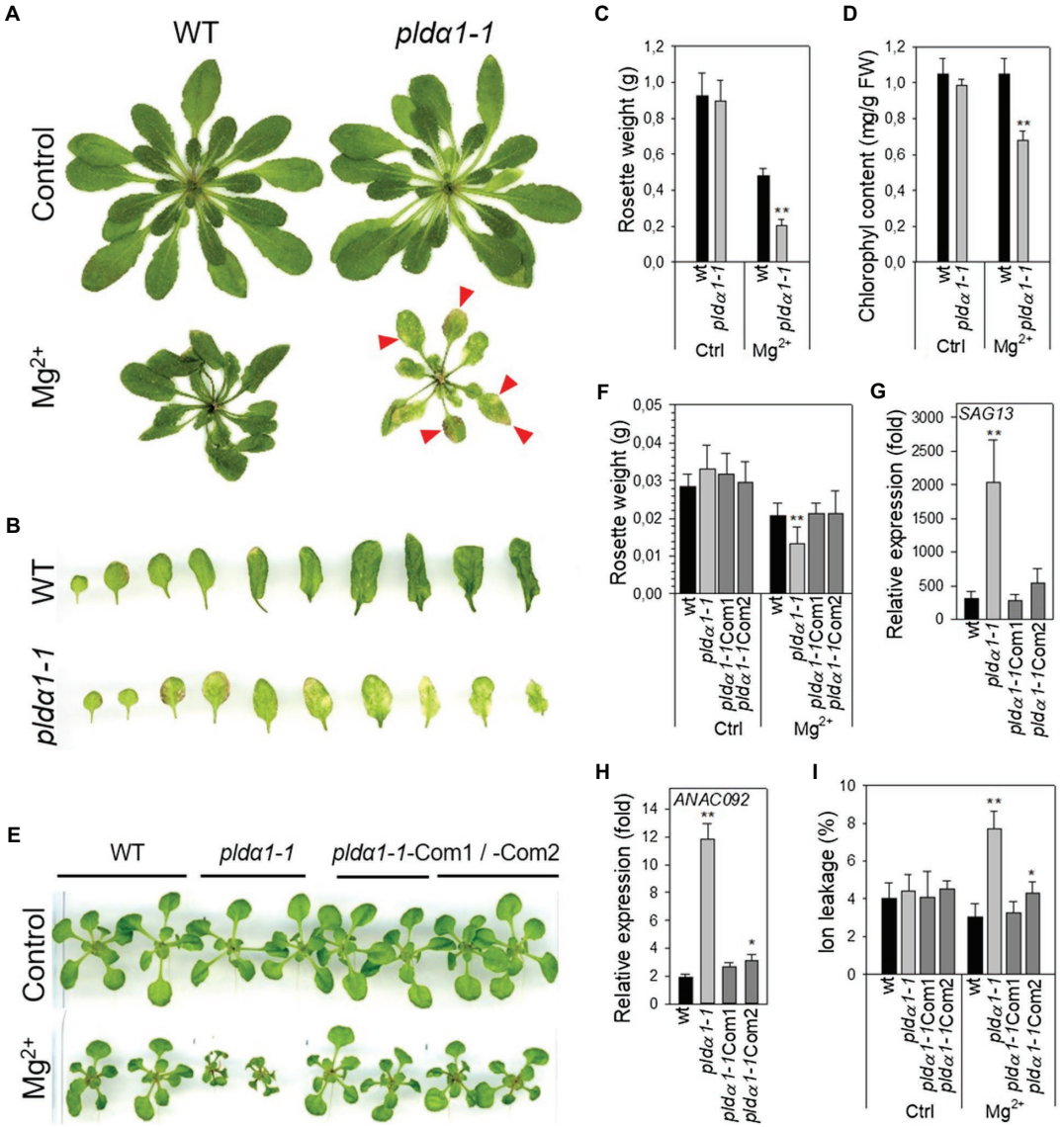

FIGURE 2 | High Mg^{2+} leads to premature senescence in *pldα1-1*. **(A)** Phenotype of WT and *pldα1-1* grown hydroponically on high Mg^{2+}, red arrows point to senescent parts of leaves. **(B)** Leaves of plants grown on high Mg^{2+}. **(C)** Weight of rosettes. **(D)** Chlorophyll content, **(A–D)** 3-week-old hydroponically grown plants were treated with 15 mM $MgSO_4$ and grown for another 16 days, values represent means ± SD, $n = 6$. **(E)** Phenotype of WT, *pldα1-1*, *pldα1-1*Com1 and *pldα1-1*Com2 plants grown on agar plates on high Mg^{2+}. **(F)** Weight of rosettes. **(G,H)** Transcript level of *SAG13* and *ANAC092* in rosettes. Transcription was normalized to a reference gene *SAND* and the transcription of non-treated plants was set to one. Values represent means ± SE, $n = 12$. **(I)** Ion leakage, values represent means ± SD, $n = 7$. **(E–I)** Ten-day-old Arabidopsis seedlings were transferred on agar plates containing 15 mM $MgCl_2$ and grown for another 7 days, Student's *t* test, asterisk indicate significant difference in comparison with WT $^*p < 0.05$; $^{**}p < 0.01$.

Supplementary Table S2). Increase of ABA as well as its catabolites phaseic acid and 9OH-ABA was more pronounced in *pldα1-1* than in WT. Interestingly, increase of cZ detected after Mg^{2+} treatment in WT was not observed in *pldα1-1*.

These results revealed that high-Mg^{2+} condition induce range of hormonal changes in both WT and *pldα1-1* plants. However, changes in ABA and JA levels observed after treatment of plants with high-Mg^{2+} were more pronounced in *pldα1-1* plants. Thus, it suggests that those hormonal changes are, at least partly, under the control of PLDα1. It means that the function of PLDα1 in regulation of hormonal changes after high-Mg^{2+} treatment is specific, as the observed difference between WT and *pldα1-1* did not affect all hormones that changed after high-Mg^{2+} treatment of plants but only ABA, JA, and cis-zeatin.

Ion Homeostasis and Levels of Starch and Proline Are Altered in *pldα1-1* Under High-Mg^{2+} Conditions

In our previous work, an imbalance of K^+ and Mg^{2+} was found in the seedlings of *pldα1-1* treated with high-Mg^{2+}. They contained

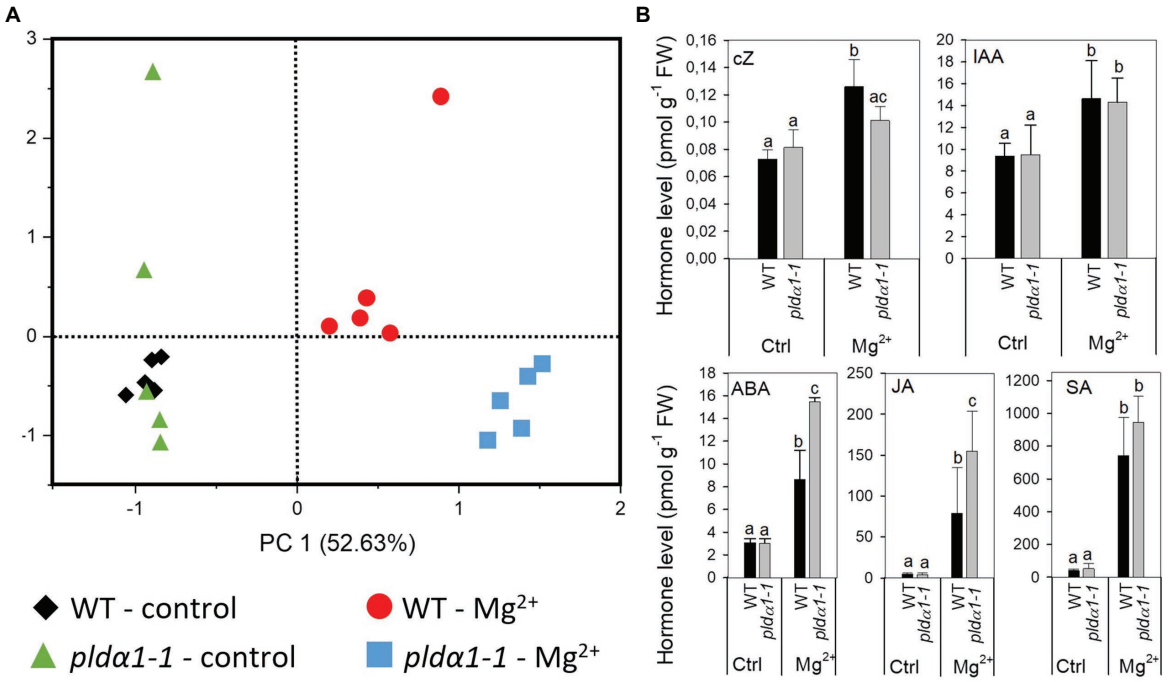

FIGURE 3 | Levels of plant hormone are altered in high-Mg^{2+} conditions. **(A)** PCA analysis. **(B)** Level of cZ, cis-zeatin; IAA, indole-3-acetic acid; ABA, abscisic acid; JA, jasmonic acid; and SA, salicylic acid. Twenty-four-day-old hydroponically grown plants were treated with 15 mM $MgSO_4$ and grown for another 2 days. Leaves 3–6 were used for hormone analysis. Values represent means ± SD, $n = 5$, letters above the bars indicate significant differences, one-way ANOVA with Tukey's *post hoc* test, $p < 0.05$.

less Mg^{2+} and K^+ under high-Mg^{2+} conditions (Kocourková et al., 2020). To reveal whether a similar ion imbalance also occurs in high-Mg^{2+}-treated shoots, we measured Mg^{2+}, Ca^{2+}, K^+, and P in WT and *pldα1-1* shoots under control and high-Mg^{2+} conditions.

After high-Mg^{2+} treatment (15 mM), Mg^{2+} content was increased approximately 5-fold in WT leaves. However, *pldα1-1* showed significantly lower Mg^{2+} content than WT (**Figure 4A**). Shoot K^+ content was lower in high-Mg^{2+}-treated plants, and *pldα1-1* plants contained even less K^+ than WT (**Figure 4B**). Ca^{2+} content was lower in high-Mg^{2+}-treated plants, but WT and *pldα1-1* content did not differ (**Figure 4C**). Furthermore, Niu et al. (2015) showed that the addition of phosphorus to high-Mg^{2+} media resulted in an increase in Arabidopsis root growth and, conversely, the addition of high-Mg^{2+} to low-P media worsened root growth. Based on these results, the authors speculated that the exacerbation of the effects of low P in the presence of high Mg^{2+} was due to the increase in the severity of P deficiency. Therefore, we also measured P content in WT and *pldα1-1* grown on high Mg^{2+} media. Remarkably, the phosphorus content in *pldα1-1* shoots under high-Mg^{2+} conditions was significantly lower than in WT (**Figure 4D**).

These results were supported by determining the expression of genes known to be associated with ion homeostasis. CBL1 is involved in potassium as well as phosphate homeostasis, whereas CIPK23 is thought to be involved in both magnesium and potassium homeostasis (Ragel et al., 2015; Tang et al., 2015; Gao et al., 2020; Sánchez-Barrena et al., 2020). In WT leaves, the expression of these genes was slightly up-regulated under high-Mg^{2+} conditions (**Figures 4E,F**). However, in *pldα1-1* leaves treated with high Mg^{2+}, *CIPK23* and *CBL1* transcripts were significantly higher than in WT plants.

Proline is well known stress molecule involved mainly in responses to drought and salt stress. Increase of proline content was also reported as response to phosphate starvation in Arabidopsis (Aleksza et al., 2017). As we observed decrease of phosphate content in high-Mg^{2+} treated *pldα1-1* plants we monitor proline level in high-Mg^{2+} treated WT and *pldα1-1* leaves. Proline content substantially increased with increasing time of Mg^{2+} treatment and was tenfold higher in WT plants treated for 7 days with high Mg^{2+} than in control plants. Interestingly, shoots of *pldα1-1* contained significantly lower level of proline after Mg^{2+} treatment in comparison with WT (**Figure 4G**).

It has been reported that that high-Mg^{2+} treatment disturbs starch homeostasis (Guo, 2014) and that both potassium and phosphorus deficiency lead to accumulation of leaf starch (Hermans et al., 2006; Hu et al., 2017). We stained starch with Lugol's solution in WT and *pldα1-1* seedlings and 24-day-old plants grown under control and high-Mg^{2+} conditions. At the end of dark period, there was clearly a higher starch accumulation in the shoot after Mg^{2+} treatment (**Figures 4H,I**). Interestingly, higher starch accumulation was observed in *pldα1-1* compared with WT plants. Moreover, shoot expression of β-amylases *BAM1* and *BAM2* was impaired in *pldα1-1* compared with WT under high Mg^{2+} treatment (**Figures 4J,K**).

These results demonstrate that Mg^{2+}, K^+, and P homeostasis, starch metabolism and proline accumulation are altered in *pldα1-1* shoots of Arabidopsis seedlings grown under high-Mg^{2+} conditions.

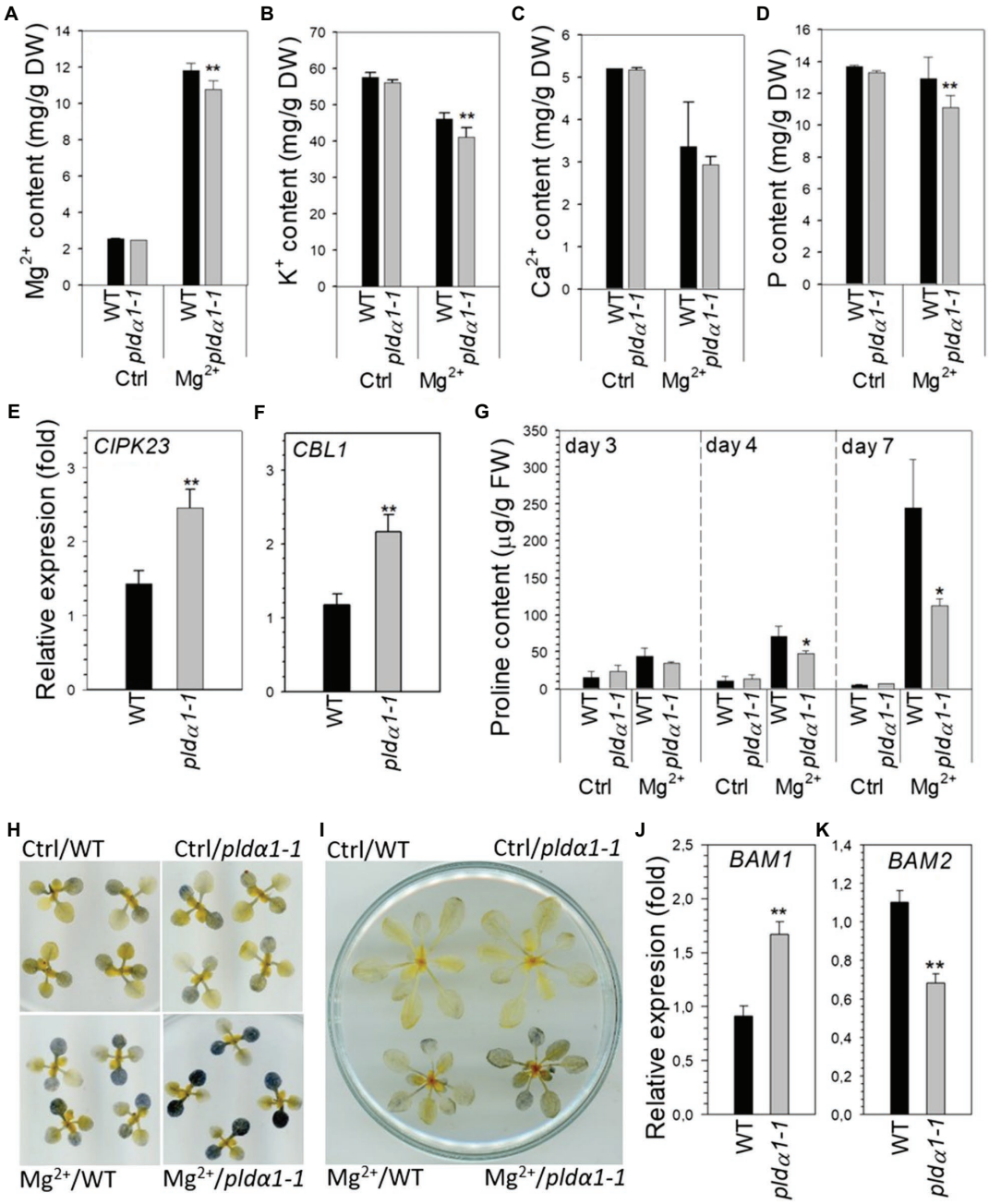

FIGURE 4 | Nutrient, proline and starch content is altered in *pldα1-1* in high-Mg^{2+} conditions. **(A–D)** Magnesium (Mg^{2+}), potassium, calcium, and phosphorus content in rosettes of WT and *pldα1-1* plants. Bars represents means ± SD, $n = 3$ for control, $n = 8$ for Mg treatment. **(E,F)** Transcript level of *CIPK23* and *CBL1* in rosettes. Transcription was normalized to a reference gene *SAND* and the transcription of non-treated plants was set to one. Values represent means ± SE, $n = 12$, **(A–F)** 10-day-old Arabidopsis seedlings were transferred on agar plates containing 15 mM $MgSO_4$ for 7 days. **(G)** Proline content, 24-day-old hydroponically grown plants were treated with 15 mM $MgSO_4$. Proline content was measured in leaves 3–6. **(H)** Lugol staining of starch in rosettes from control and high Mg^{2+} condition at the end of dark period. Ten-day-old Arabidopsis seedlings were transferred on agar plates containing 15 mM $MgSO_4$ for 3 days. **(I)** Lugol staining of starch in rosettes from control and high Mg^{2+} condition at the end of dark period, 24-day-old hydroponically grown plants were treated with 15 mM $MgSO_4$ for 3 days. **(J,K)** Transcript level of *BAM1* and *BAM2* in leaves 3–6, 24-day-old hydroponically grown plants were treated with 15 mM $MgSO_4$ for 2 days, transcription was normalized to a reference gene *SAND* and the transcription of non-treated plants was set to one. Values represent means ± SE, $n = 12$. Student's *t* test, asterisk indicate significant difference in comparison with WT $^*p < 0.05$; $^{**}p < 0.01$.

DISCUSSION

In our previous work (Kocourková et al., 2020) we found that *pldα1* plants have shorter roots under high Mg^{2+} conditions compared to WT. We also showed that PLDα1 activity contributes significantly to tolerance to high Mg^{2+}. In this work, we focused on the shoots. Our original hypothesis was that PLDα1 activity is important mainly in roots, as they are exposed to high

Mg^{2+} conditions and an increase in PLD activity is rapidly induced after high Mg^{2+} treatment (Kocourková et al., 2020). However, we found that PLDα1 activity in the aerial parts of WT Arabidopsis also increased after treatment of the plants with high level of Mg^{2+} ions. Using western blots, we also showed that the amount of PLDα1 increased in shoots treated with high Mg^{2+} and that *pldα1* plants exhibited premature leaf senescence under high Mg^{2+} conditions. Thus, PLDα1 appears to act as a negative regulator of senescence induced by high Mg^{2+}.

Magnesium-Induced Senescence

Leaf senescence is a highly coordinated process. In addition to age-dependent senescence, there is also stress-induced senescence caused by abiotic (drought, salt, high or low temperature, and nutrient imbalance) and biotic stresses (Sade et al., 2018; Guo et al., 2021). Leaf senescence is associated with membrane and chlorophyll degradation. Leaf yellowing and senescence have been reported to be induced by magnesium deficiency (for a review, see Tanoi and Kobayashi, 2015). In addition, a decrease in chlorophyll content was observed by Yan et al. (2018) under conditions of Mg^{2+} imbalance (Mg deficiency and excess). We observed a greater decrease in leaf chlorophyll content and higher ion leakage in *pldα1* plants than in WT. Also, the transcript level of the senescence marker genes *SAG13* (Dhar et al., 2020) and *ANAC092* (Weaver et al., 1998; Miller et al., 1999; John et al., 2001) was significantly higher in *pldα1* plants, although the expression of both genes was also increased in WT plants in which no signs of senescence were yet evident. Since both genes are among the markers of the onset of senescence, it can be concluded that senescence processes are also initiated in WT upon high Mg^{2+} treatment. However, plants with dead PLDα1 tolerate the stress caused by high Mg^{2+} concentrations much worse than WT, leading to apparent premature leaf senescence.

The High Mg^{2+} Induced Senescence-Associated Hormonal Changes

Hormones play a critical role in regulating both development and stress-induced senescence. Cytokinins, auxin and gibberellic acid (GA) delay leaf senescence, while ABA, salicylic acid (SA), JA, ethylene and strigolactones (SL) promote leaf senescence (Lim et al., 2007; Guo et al., 2021). The overall hormonal changes we observed after high Mg^{2+} treatment of WT plants were in good agreement with the reported hormonal changes during leaf senescence. We found that after 2 days of high Mg^{2+} treatment, there was a decrease in active CK, such as *trans*-zeatin (tZ) and its riboside (tZR) which is in line with gradual decrease in cytokinin content observed during leaf senescence (Singh et al., 1992; Gan and Amasino, 1996). On the other hand, the content of the stress-related CKs *cis*-zeatin (cZ), its riboside (cZR), and phosphate (cZRMP) increased. An increase in cZ during natural senescence was reported in Arabidopsis and tobacco (Gajdošová et al., 2011; Uzelac et al., 2016). The level of cZ differed in WT and *pldα1* plants under high Mg^{2+} conditions. This suggests that the level of cZ is regulated by PLDα1 under high Mg^{2+} conditions. However, the level of cZ in more senescent *pldα1* plants is lower compared with WT. This is a counterintuitive finding, and further studies are required to clarify this phenomenon.

Abscisic acid is a plant hormone whose level increases significantly after abiotic stresses such as drought and salt stress. During leaf senescence, the level of ABA increases, and exogenous application of ABA induces leaf senescence (Lim et al., 2007; Zhang et al., 2012). We also observed a significant increase in ABA level after high Mg^{2+} treatment in WT and *pldα1* plants, while the increase of ABA was higher in *pldα1* plants than in WT plants. This is consistent with the higher senescence of *pldα1* induced by high Mg^{2+} content, which is also consistent with the observations of Guo et al. (2014). The authors found an increase in ABA content after long-term (14 days) exposure of Arabidopsis to high Mg^{2+}. They also showed that ABA insensitive plants *abi1-1* were less sensitive to high Mg^{2+} treatment. In our experiments, a significant ABA response was observed after only 48 h of exposure. Moreover, transcriptome analysis of Arabidopsis roots treated with high Mg^{2+} revealed increased expression of 9-cis-epoxycarotenoid dioxygenase, an enzyme associated with the biosynthesis of ABA, after 45 min of high Mg^{2+} treatment (Visscher et al., 2010). All these results suggest that the increase in ABA content and subsequent ABA signaling are involved in the PLDα1-mediated early responses to high Mg^{2+} conditions.

Salicylic acid (SA) and JA are hormones known primarily for their involvement in plant defence mechanisms against pathogens. However, they are also associated with many responses to abiotic stresses (Miura and Tada, 2014; Raza et al., 2020). It has been shown that the level of SA increases progressively during leaf senescence (Breeze et al., 2011; Zhang et al., 2017) and SA plays a direct role in the onset and progression of leaf senescence (Guo et al., 2021). JA content increases during both natural and induced leaf senescence, and external application of JA induces leaf senescence (He et al., 2002; Seltmann et al., 2010). In our experiments, a significant increase in SA and JA was observed in both WT and *pldα1* plants after high Mg^{2+} treatment, and this increase was more pronounced in *pldα1* plants.

JA signaling has been shown to play a role in the biosynthesis of camalexin (Pangesti et al., 2016), a phytoalexin with a described role in the defence response to a variety of pathogens. Its biosynthesis is also induced by some abiotic treatments such as ROS-inducing compound acifluorfen (Zhao et al., 1998) or UV-B irradiation (Mert-Turk et al., 2003). Interestingly, camalexin content increased after high Mg^{2+} treatment in WT and even more (by 4.5-fold) in *pldα1* plants (**Supplementary Table S2**). It is not clear what role camalexin might play in response to high Mg^{2+} treatment. However, higher camalexin levels in *pldα1* plants might be related to higher JA levels in *pldα1* plants treated with high Mg^{2+}. In addition, camalexin biosynthesis is regulated by MPK6 kinase, which has been shown to be a PA binding protein (Yu et al., 2010).

Ion Imbalance, Starch and Proline Content and Their Role in Senescence

We found changes in ion content in seedlings (Kocourková et al., 2020) and leaves (this work) of plants treated with high Mg^{2+}. At WT, the K^+ content of plants treated with high Mg^{2+}

was lower compared to untreated controls. Moreover, K^+ content under high Mg^{2+} conditions was significantly lower in *pldα1* plants than in WT. Similarly, *pldα1* and WT plants also differed in P content under high-Mg^{2+} conditions; *pldα1* plants had lower P contents than WT. However, the P content of WT did not differ between control and high-Mg^{2+} conditions.

Potassium deficiency has been reported to induce leaf senescence in Arabidopsis and cotton. Interestingly, there is also strong evidence that JA is involved in potassium deficiency – induced leaf senescence (Armengaud et al., 2004; Cao et al., 2006; Li et al., 2012; Hu et al., 2016). We demonstrated an increase in JA level after high Mg^{2+} treatment in both WT and *pldα1*, and that the accumulation of JA in *pldα1* was higher than that of WT. Further experiments are needed to determine whether potassium deficiency, JA accumulation, and leaf senescence are related.

Leaf starch is synthesized during the day and mobilized during the following night to provide a steady supply of carbon and energy. Starch also mediates plant responses to abiotic stresses such as water deficit, high salinity or extreme temperatures. Most studies considered that starch content in leaves decreases in response to abiotic stresses. However, there are also reports that starch accumulation increases in Arabidopsis under stress (Kaplan and Guy, 2004; Skirycz et al., 2009). In our work, we observed increased starch accumulation under high Mg^{2+} conditions in WT plants, and starch accumulation was even higher under these conditions in *pldα1* plants. The opposite effect of high Mg^{2+} was observed by Guo et al. (2014). The authors found lower leaf starch level in Arabidopsis WT under high Mg^{2+} conditions than under control conditions. It is not clear why such different results occurred. One possible explanation could be the use of different ecotypes and experimental conditions. Guo et al. (2014) used an ecotype (Landsberg erecta), a higher Mg^{2+} concentration (32 mM) and long-term high Mg^{2+} stress, while we observed starch in leaves on the third day after treating Arabidopsis plants of ecotype Columbia 0 with 15 mM Mg^{2+}. The relationship between high starch accumulation and leaf senescence has also been described (Schaffer et al., 1991; Oda-Yamamizo et al., 2016; Huang et al., 2018; Xiao et al., 2020). A possible link between PLDα1 and altered starch accumulation could be the PA binding protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH; McLoughlin et al., 2013), as seedlings with genetically reduced GAPDH activity accumulated higher amounts of starch compared to WT (Yang et al., 2015). Moreover, phosphorus deficiency increases leaf sugars and starch content (Cakmak et al., 1994; Hermans et al., 2006) and we found lower phosphorus content in the leaves of *pldα1* plants compared to WT.

We observed proline accumulation in plants exposed to high Mg^{2+} conditions. Proline is a well-known molecule involved in adaptation to stress by, e.g., balancing cellular redox potential, scavenging free radicals and stabilizing subcellular structures (Szabados and Savoure, 2009; Kaur and Asthir, 2015). A relationship between proline metabolism and leaf senescence has been previously noted and discussed (Zhang and Becker, 2015). Proline content significantly increased in detached rice leaves during senescence (Wang et al., 1982). On the other hand, proline catabolism appears to be up-regulated in Arabidopsis during natural leaf senescence (Funck et al., 2010). Moreover, experiments with inhibition of PLD activity by 1-butanol during salt stress showed that PLD appears to be a negative regulator of the delta-1-pyrroline-5-carboxylate synthase 1 gene, which controls proline biosynthesis (Thiery et al., 2004). We have demonstrated that exposure of *pldα1* to high Mg^{2+} resulted in decreased proline accumulation compared to WT. Thus, PLDα1 appears to be a positive regulator of proline synthesis. Why PLDα1-depleted plants have less proline under high-Mg^{2+} conditions is unclear. The difference in proline content between WT and *pldα1* is significant only after prolonged exposure (4 days) to high Mg^{2+}, and it is therefore possible that this is a side effect of earlier changes caused by high Mg^{2+} rather than a direct regulation of proline metabolism by PLDα1. However, it is possible that proline helps the plants to cope with the stress caused by high Mg^{2+}, and the lower proline content of *pldα1* may contribute to the manifestation of senescence in plants with dysfunctional PLDα1. Interestingly, significant differences in proline content were observed between WT and phosphoenolpyruvate carboxylase 3 (PEPC3) knockout in Arabidopsis under control and salt stress conditions and PEPC3 was identified as the PA-binding protein (Testerink et al., 2004).

Mechanism of PLDα1 Involvement in Leaf Senescence

PLDα1 has been described to be involved in a variety of biological processes. PLDα1 knockout or antisense-suppressed plants show alterations in water loss, reactive oxygen species production (ROS), response to ABA and stomatal movement (Zhang et al., 2004, 2009; Mishra et al., 2006), salt stress (Bargmann et al., 2009; Yu et al., 2010, 2015), freezing sensitivity (Rajashekar et al., 2006) and seed aging (Devaiah et al., 2007). The involvement of PLDα1 in senescence has also been documented. In 1997, Fan et al. (1997) observed that treatment of detached leaves with ABA and ethylene led to accelerated senescence and increased level of PLDα mRNA, protein and activity. Using the PLDα antisense construct, they then prepared plants with reduced PLDα1 expression. Suppression of PLDα had no effect on natural plant growth and development. Even in the absence of ABA and ethylene, the detached leaves of the PLDα-deficient and WT plants showed similar rate of senescence.

However, the senescence rate of detached leaves of transgenic plants treated with ABA or ethylene was slower than that of detached leaves from WT. Later, Jia et al. (2013) showed that the application of n-butanol, an inhibitor of PLD, and N-acylethanolamine (NAE) 12:0, a specific inhibitor of PLDα, delayed ABA-promoted senescence to different extents. These data suggest that suppression of PLDα blocks membrane lipid degradation, which ultimately delays ABA-promoted senescence. Thus, PLDα1 appears to be important mediator that play a positive role in phytohormone-promoted senescence in detached leaves. However, in this work, we showed that PLDα1 likely serves as a negative regulator of senescence. We observed that PLDα1 activity and PLDα1 abundance increase during senescence

triggered by high Mg^{2+} content. An increase in PLDα1 expression has also been described during age-related leaf senescence (Xiao et al., 2010). However, in our case, the increase in PLDα1 activity and PLDα1 abundance is probably not related to the increased membrane degradation described above, because *pldα1-1* plants exhibited significantly higher senescence compared with WT. Therefore, another regulatory mechanism by which PLDα1 is involved in the regulation of senescence induced by high Mg^{2+} levels must play a role.

In general, there are two molecular ways by which PLDα1 may regulate other events. The first is linked with PLDα1 activity which leads to the production of the second messenger phosphatidic acid and free head group, and the second is protein–protein interaction. In the case of PLDα1, both scenarios have been documented. A combination of both mechanisms is also possible and has been described in the case of PLDα1 involvement in ABA responses (see below). PLD-derived phosphatidic acid is produced in response to various biotic and abiotic stresses such as plant defence, wounding, salt, drought, cold, and heat stress (Yao and Xue, 2018). In salt stress PA produced by activated PLDα1 binds to ABI1, a protein phosphatase 2C, a negative regulator of the ABA response and inhibits its function (Zhang et al., 2004). Since ABA functions as a positive regulator of leaf senescence, PLDα1 could also play the role of a positive regulator of leaf senescence. However, in our study, this is not the case because PLDα1 is a negative regulator of leaf senescence induced by high Mg^{2+} content.

A number of PA-binding proteins have been found (Yao and Xue, 2018). CTR1 (CONSTITUTIVE TRIPLE RESPONSE1) is another example of PA binding protein (Testerink et al., 2004, 2007). CTR1 is a Ser/Thr protein kinase that functions as a negative regulator of ethylene signaling. Loss of CTR1 function has been shown to promote the senescence process upon dark treatment, suggesting that CTR1 plays a role as a negative regulator of leaf senescence (Li et al., 2017). We did not measure ethylene levels in our experimental setup. However, ethylene is known to be an endogenous modulator of senescence, including leaf senescence.

PLDα1 protein interaction, the second possible PLDα1 regulatory mechanism, is also involved in the regulation of ABA responses. PLDα1 interacts with components of heterotrimeric G protein signaling, GPA1 (Gα) and Gβ proteins (Zhao and Wang, 2004; Gookin and Assmann, 2014). The interaction of PLDα1 with GPA1 stimulates the GTPase activity of GPA1 (Zhao and Wang, 2004). PLDα1 also interacts with RGS1 protein (regulator of G protein signaling). RGS1 likely inhibits the GAP activity of PLDα1 (Choudhury and Pandey, 2016). To further impact the specificity of this pathway, PA, the product of PLDα1 activity, binds to RGS1 and inhibits its GAP activity. Interestingly, GPA1-, Gβ- as well as RGS1 knock-out plants showed altered salt stress-induced senescence (Colaneri et al., 2014).

In summary, high Mg^{2+} induces leaf senescence and many of the physiological changes associated with leaf senescence induced by high Mg^{2+} are under the control of PLDα1. Subsequent studies should elucidate the precise molecular mechanism of this PLDα1 control.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DK and JM designed the study and wrote the manuscript. DK, KK, TP, and MD performed the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.770794/full#supplementary-material

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REVIEW
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Multiple Layers of Regulation on Leaf Senescence: New Advances and Perspectives

*Yue-Mei Zhang[1], Pengru Guo[1], Xinli Xia[1], Hongwei Guo[2] and Zhonghai Li[1]**

[1] National Engineering Laboratory for Tree Breeding, College of Biological Sciences and Technology, Beijing Forestry University, Beijing, China, [2] Key Laboratory of Molecular Design for Plant Cell Factory of Guangdong Higher Education Institutes, Department of Biology, Southern University of Science and Technology, Shenzhen, China

Leaf senescence is the last stage of leaf development and is an orderly biological process accompanied by degradation of macromolecules and nutrient recycling, which contributes to plant fitness. Forward genetic mutant screening and reverse genetic studies of senescence-associated genes (SAGs) have revealed that leaf senescence is a genetically regulated process, and the initiation and progression of leaf senescence are influenced by an array of internal and external factors. Recently, multi-omics techniques have revealed that leaf senescence is subjected to multiple layers of regulation, including chromatin, transcriptional and post-transcriptional, as well as translational and post-translational levels. Although impressive progress has been made in plant senescence research, especially the identification and functional analysis of a large number of SAGs in crop plants, we still have not unraveled the mystery of plant senescence, and there are some urgent scientific questions in this field, such as when plant senescence is initiated and how senescence signals are transmitted. This paper reviews recent advances in the multiple layers of regulation on leaf senescence, especially in post-transcriptional regulation such as alternative splicing.

Keywords: leaf senescence, senescence-associated genes, multi-omics, gene regulatory network, alternative splicing

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****Correspondence:***
Zhonghai Li
lizhonghai@bjfu.edu.cn

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INTRODUCTION

Plant leaves are the main organ for photosynthesis, converting light energy into chemical energy stored in carbohydrate molecules, which is the main source of energy for all organisms on earth. Senescence is the final stage of leaf development process, which is a slow and complex biological process including the initiation, progression, and terminal phases (Guo and Gan, 2005; Lim et al., 2007). The degradation of chlorophyll and chloroplasts occurs in the later phase of leaf senescence, accompanied by the degradation of macromolecules such as proteins, lipids, and nucleic acids. In annual plants, the nutrients released from senescent leaves are transferred to actively growing young leaves and seeds to increased reproductive success. In perennial plants such as deciduous trees, the nitrogen from leaf proteins is relocated to form bark storage proteins (BSP) in phloem tissues, and then remobilized and reutilized for spring shoot growth. Therefore, the timing of leaf senescence plays an important role in ensuring nutrient recycling, adaptation to the environment, and reproduction in plants. A number of studies in crops such as wheat and rice revealed that alteration of leaf senescence process could significantly affect the yield and quality of crops.

Extended lifespan of leaves in apple trees greatly improved fruit quality in apple trees (Han et al., 2020; Hu et al., 2020), and increased fruit yield and sugar content in tomato (*Solanum lycopersicon*) (Lira et al., 2017; Ma et al., 2018). Moreover, delayed leaf senescence conferred enhance drought resistance in tobacco or cassava (Zhang et al., 2010). Therefore, an in-depth understanding of the regulatory mechanisms of leaf senescence is of great importance.

Leaf senescence is not a passive and disorderly process, but a highly programmed degenerative process (Guo and Gan, 2005). The initiation and progression of leaf senescence are influenced by numerous endogenous developmental signals and external environmental factors. Leaf age is the most important endogenous cue that determines the initiation of leaf senescence. However, the nature of age and how age information is perceived remains a mystery (Jing et al., 2002). Plant hormones such as ethylene, jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), brassinosteroid (BR), and strigolactone (SL) promote leaf senescence and are extensively involved in response to various abiotic and biotic stresses, whereas auxin, cytokinins (CKs), and gibberellins (GAs) delay leaf senescence (Lim et al., 2007; Miao and Zentgraf, 2007; Li et al., 2013; Zhang et al., 2013; Yamada and Umehara, 2015; Kim et al., 2020). Hormone signaling pathways often mediate or influence development and environmental responses to regulate leaf senescence (Lim et al., 2007). Interestingly, changes in the circadian rhythm of plants also impact leaf senescence, but the causal relationship between them needs to be further explored (Song et al., 2018). In addition to being regulated by plant age or phytohormones, leaf senescence can also be caused by numerous environmental stresses such as darkness, nutrient deficiency, drought stress, and pathogen infection (Lim et al., 2007; Chen et al., 2013; Woo et al., 2019; Li et al., 2020b). There is much information about age- or abiotic stress-induced leaf senescence, whereas little is known about the molecular basis of biotic stress-triggered senescence. Recently, it was found that the secretory effector protein PevD1 (Protein elicitor from *Verticillium. dahliae* 1) plays an important role in the *V. dahliae*–induced senescence process. PevD1 interacts with ORESARA1 (ORE1), one core transcription factor regulating plant senescence (Lim et al., 2007), and attenuates the NLA-mediated degradation of ORE1, thereby enhancing ethylene biosynthesis by directly binding the promoter of 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6 (ACS6) (Zhang Y. et al., 2021). This research provides a mechanism for previous observations that ethylene contributes to *V. dahliae*–induced premature leaf senescence.

MULTIPLE-LAYERS OF REGULATION ON LEAF SENESCENCE

In the past few decades, remarkable progress has been made in leaf senescence research, and time-evolving genetic networks have been established through genetics and multi-omics strategies, allowing us to gain a deeper understanding of this important biological process (Kim H. J. et al., 2018).

Here, we reviewed the recent advances in the molecular regulation of leaf senescence, including chromatin level, transcription level, as well as post-transcriptional, translational, and post-translational level (**Figure 1**). We also summarized the key players involved in the multilevel regulation of leaf senescence (**Table 1**).

Chromatin Level

In eukaryotic cells, DNA is packaged into chromatin and its functional units are nucleosomes. The basic unit of chromatin is the nucleosome core particle, a structure in which ∼146 bp of DNA is wrapped around a protein octamer consisting of two subunits each of core histones H2A, H2B, H3, and H4 (Luger et al., 1997; Davey et al., 2002; Marino-Ramirez et al., 2005). The globular region of the histone forms the core of the nucleosome, while the N-terminal tail protrudes from the nucleosomes and is enriched for various post-translational modifications (PTMs), including acetylation, methylation, phosphorylation, and ubiquitination (Bannister and Kouzarides, 2011). These modifications have important regulatory roles, including gene repression, gene activation, and replication (Kouzarides, 2007; Morgan and Shilatifard, 2020). Histone modifications and the enzymes that implement them can facilitate chromatin compaction, nucleosome dynamics, and transcription. These modifications can respond to intrinsic and external stimuli (Kouzarides, 2007). Dysregulation of these processes can alter the balance of gene expression and thus is often observed in many human diseases or plant development, either by gain or loss of function, overexpression, repression through promoter hypermethylation, chromosomal translocation, or mutation of histone-modifying enzymes/complexes, or even histone modification sites (Zhao and Shilatifard, 2019).

Previous investigations revealed that epigenetic modification participates in the plant leaf senescence process. Chromatin immunoprecipitation sequencing (ChIP-seq) analysis using the trimethylation of histone H3 at lysine 4 (H3K4me3) and the trimethylation of histone H3 at lysine 27 (H3K27me3) antibodies reveals the relationship between histone modifications and leaf senescence in *Arabidopsis* (Brusslan et al., 2012, 2015). Mutation of histone deacetylase *AtHD1*, a histone modification-related gene, altered leaf senescence process in *Arabidopsis* (Pandey et al., 2002). The histone acetylation status of specific parts of chromatin is determined by histone acetylases (HATs) and histone deacetylases (HDACs) and their relative activities. Histone acetyltransferase 1 (HAC1) promotes leaf senescence by targeting Ethylene-responsive transcription factor ERF022, a positive regulator of leaf senescence (Hinckley et al., 2019). HISTONE DEACETYLASE 9 (HDA9), HDA15, HDA19, HISTONE DEACETYLASE 2C (HD2C), and SIRTUIN 1 (SRT1) play a potential role in promoting leaf senescence (Buszewicz et al., 2016; Zheng et al., 2016; Liu et al., 2017; Ueda et al., 2018; Hu et al., 2019; Shen et al., 2019). ChIP-seq and fluorescence *in situ* hybridization (FISH) reveal that the chromatin structure changes as the leaf ages in *Arabidopsis*. Overexpression of *SUVH2*, a SU(VAR)3-9 (KMTase1) histone methyltransferase gene, delayed leaf senescence by increasing

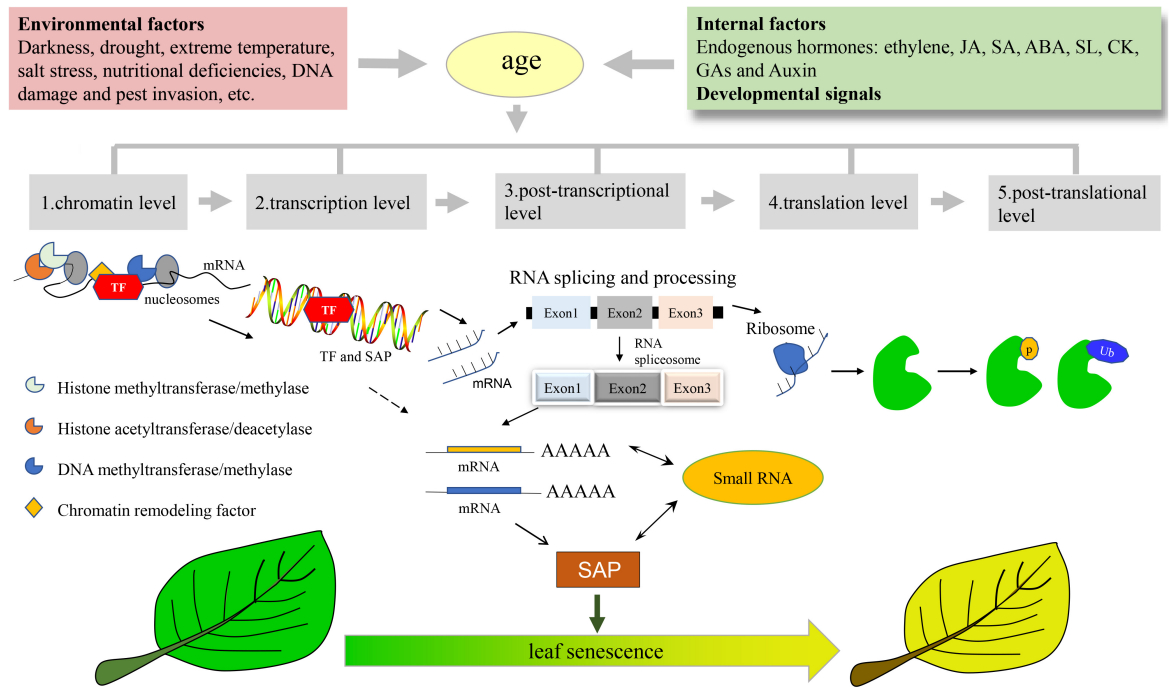

FIGURE 1 | Multiple Layers of Regulation on Plant Leaf Senescence. Plant leaf senescence is finely regulated by endogenous developmental signals, and external environmental cues in an age-dependent manner. The multiple layers of regulation on leaf senescence includes epigenetic regulation at the chromatin level involved in histone proteases, DNA methylation modifying enzymes and chromatin remodeling factors, the master transcription factors involved in transcription regulation such as WRKYs and NACs, also includes miRNA and alternative splicing involved in the post-transcriptional level, and translation initiation and elongation factors involved in the regulation of translation level, as well as post-translational ubiquitination and phosphorylation. SAP, senescence-associated protein; Ub, ubiquitin; TF, transcription factors.

the dimethylation of histone H3 at lysine 27 (H3K27me2) and H3K27me3 levels in the promoter region of *WRKY53*, a master positive regulator of leaf senescence, and then suppressing its transcripts (Ay et al., 2009). JUMONJI DOMAIN-CONTAINING PROTEIN 16 (JMJ16), a specific H3K4 demethylase containing JmjC-domain, regulates negatively leaf senescence through repressing the expression of *WRKY53* and *SENESCENCE-ASSOCIATED GENE 201* (*SAG201*), two positive regulators of leaf senescence in *Arabidopsis*. Moreover, genome-wide analysis reveals widespread hypermethylation of H3K4me3 at JMJ16 binding genes, including *WRKY53* and *SAG201*, and coordinated upregulation of their expression in the *jmj16* mutant compared with the wild type (Liu et al., 2019). To screen the upstream regulator of NONYELLOWING1 (NYE1) that regulates chlorophyll degradation during leaf senescence by Yeast one-Hybrid (Y1H) approach, the histone H3K27me3 demethylase RELATIVE OF EARLY FLOWERING6 (REF6) was found to directly interact with the NYE1/2 promoter through its zinc finger domain and up-regulates gene expression of positive regulators of leaf senescence such as *ETHYLENE INSENSITIVE 2* (*EIN2*) and *ORE1* (Wang et al., 2019). In addition, overexpression of *SUVH2* also inhibits the gene expressions of senescence-associated WRKY (Sen-WRKY) and Sen-NAC (NAM/ATAF/CUC) transcription factors, central components of the leaf senescence process, in *Arabidopsis* leaves upon treatment with bleomycin (BLM), a genotoxic chemical that induces double-strand breaks (DSBs) (Li et al., 2020a).

ATP-dependent chromatin remodeling enzyme involved in chromatin remodeling is also associated with leaf senescence. Mutations of *DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1* (*DRD1*) and *DECREASED DNA METHYLATION 1* (*DDM1*), two SWI2/SNF2 chromatin remodeling proteins, delay leaf senescence (Cho et al., 2016). In contrast, loss-of-function of *BRAHMA* (*BRM*), another SWI/SNF2 chromatin remodeling ATPase (Archacki et al., 2017), accelerates leaf senescence (Efroni et al., 2013; Li C. et al., 2016). Further studies are needed in the future to reveal how various epigenetic modifications coordinately regulate leaf senescence.

Transcription Level

Large-scale reprogramming of the transcriptome is a core step in plant leaf senescence. Approximately a dozen percent of genes are up-regulated or down-regulated during leaf senescence in *Arabidopsis*. Furthermore, master TFs-mediated transcriptional regulation plays a crucial role in the regulation of leaf senescence (Zentgraf et al., 2010; Breeze et al., 2011). A WRKY transcription factor is one of the plant-specific TF families controlling the leaf senescence process. Members of WRKY TFs, including WRKY6, WRKY22, WRKY42, WRKY45, WRKY46, WRKY53, WRKY54, WRKY55, WRKY57, WRKY70, and WRKY75, coordinate with endogenous hormones to finely regulate the leaf senescence

TABLE 1 | List of the key genes involved in multiple layers of regulation on leaf senescence.

Gene	Species	Effects	Regulation	References
AtHD1	*Arabidopsis thaliana*	Delay	Chromatin level	Pandey et al., 2002
AtSRT1	*Arabidopsis thaliana*	Delay	Chromatin level	Liu et al., 2017
BRAHMA	*Arabidopsis thaliana*	Delay	Chromatin level	Efroni et al., 2013
DRD1/DDM1	*Arabidopsis thaliana*	Promote	Chromatin level	Cho et al., 2016
HAC1	*Arabidopsis thaliana*	Promote	Chromatin level	Hinckley et al., 2019
HDA9	*Arabidopsis thaliana*	Delay	Chromatin level	Zheng et al., 2016
HDA15	*Arabidopsis thaliana*	Delay	Chromatin level	Shen et al., 2019
HDA19	*Arabidopsis thaliana*	Delay	Chromatin level	Ueda et al., 2018
HD2C	*Arabidopsis thaliana*	Delay	Chromatin level	Buszewicz et al., 2016
JMJ16	*Arabidopsis thaliana*	Promote	Chromatin level	Liu et al., 2019
SUVH2	*Arabidopsis thaliana*	Delay	Chromatin level	Ay et al., 2009
REF6	*Arabidopsis thaliana*	Promote	Chromatin level	Wang et al., 2019
WRKY6	*Arabidopsis thaliana*	Promote	Transcription level	Robatzek and Somssich, 2001
WRKY22	*Arabidopsis thaliana*	Promote	Transcription level	Zhou et al., 2011
WRKY42	*Arabidopsis thaliana*	Promote	Transcription level	Niu et al., 2020
WRKY45	*Arabidopsis thaliana*	Promote	Transcription level	Chen et al., 2017
WRKY46	*Arabidopsis thaliana*	Promote	Transcription level	Zhang D. et al., 2021
WRKY53	*Arabidopsis thaliana*	Promote	Transcription level	Zentgraf et al., 2010
WRKY54/WRKY70	*Arabidopsis thaliana*	Delay	Transcription level	Besseau et al., 2012
WRKY55	*Arabidopsis thaliana*	Promote	Transcription level	Wang et al., 2020
WRKY57	*Arabidopsis thaliana*	*Delay*	*Transcription level*	Jiang et al., 2014
WRKY75	*Arabidopsis thaliana*	Promote	Transcription level	Guo P. et al., 2017
GhWRKY42	*Gossypium hirsutum*	Promote	Transcription level	Gu et al., 2018
GhWRKY91	*Gossypium hirsutum*	Delay	Transcription level	Gu et al., 2019
CpWRKY71	*Chimonanthus praecox*	Promote	Transcription level	Huang et al., 2019
OsWRKY42	*Oryza sativa*	Promote	Transcription level	Han et al., 2014
OsWRKY93	*Oryza sativa*	Promote	Transcription level	Li Y. et al., 2021
BnaWSR1	*Brassica napus*	Promote	Transcription level	Cui et al., 2020
BnaWGR1	*Brassica napus*	Promote	Transcription level	Yang et al., 2018
BrWRKY6	*Brassica rapa var. parachinensis*	Promote	Transcription level	Fan et al., 2018
AtNAP	*Arabidopsis thaliana*	Promote	Transcription level	Guo and Gan, 2006
AtNAC3	*Arabidopsis thaliana*	Promote	Transcription level	Hickman et al., 2013
ATAF1	*Arabidopsis thaliana*	Promote	Transcription level	Garapati et al., 2015
ANAC016	*Arabidopsis thaliana*	Promote	Transcription level	Kim et al., 2013
ANAC017, VNI1, and ANAC090	*Arabidopsis thaliana*	Delay	Transcription level	Woo et al., 2016
ANAC019	*Arabidopsis thaliana*	Promote	Transcription level	Lee et al., 2015
ANAC032	*Arabidopsis thaliana*	Promote	Transcription level	Mahmood et al., 2016
ANAC046	*Arabidopsis thaliana*	*Promote*	*Transcription level*	Oda-Yamamizo et al., 2016
ANAC072	*Arabidopsis thaliana*	*Promote*	*Transcription level*	Li S. et al., 2016
NAC075	*Arabidopsis thaliana*	Delay	Transcription level	Kan et al., 2021
ANAC102	*Arabidopsis thaliana*	Promote	Transcription level	Nakashima et al., 2012
JUB1	*Arabidopsis thaliana*	Delay	Transcription level	Wu et al., 2012
NTL9	*Arabidopsis thaliana*	*Promote*	*Transcription level*	Yoon et al., 2008
ORE1	*Arabidopsis thaliana*	Promote	Transcription level	Kim et al., 2009
ORS1	*Arabidopsis thaliana*	Promote	Transcription level	Balazadeh et al., 2011
PIF4 and PIF5	*Arabidopsis thaliana*	Promote	Transcription level	Sakuraba et al., 2014; Song et al., 2014
VNI2	*Arabidopsis thaliana*	Delay	Transcription level	Yang et al., 2011
BnaNAC87	*Brassica napus*	Promote	Transcription level	Yan et al., 2018
IbNAC1	*Ipomoea batatas*	Promote	Transcription level	Chen et al., 2016
MpSNAC67	*Musa x paradisiaca*	Promote	Transcription level	Tak et al., 2018
MlNAC5	*Miscanthus lutarioriparius*	Promote	Transcription level	Yang et al., 2015
NtNAC080	*Nicotiana tabacum*	Promote	Transcription level	Li et al., 2018
NAM-B1	*Triticum turgidum*	Promote	Transcription level	Uauy et al., 2006

(Continued)

TABLE 1 | (Continued)

Gene	Species	Effects	Regulation	References
TaNAC-S	*Triticum turgidum*	Delay	Transcription level	Zhao et al., 2015
ONAC011	*Oryza sativa*	Promote	Transcription level	El Mannai et al., 2017
ONAC096	*Oryza sativa*	Delay	Transcription level	Kang et al., 2019
ONAC106	*Oryza sativa*	Delay	Transcription level	Sakuraba et al., 2015
OsNAC2	*Oryza sativa*	Promote	Transcription level	Mao et al., 2017
OsNAP	*Oryza sativa*	Promote	Transcription level	Liang et al., 2014
OsDOS	*Oryza sativa*	Delay	Transcription level	Kong et al., 2006
SlNAP1 and SlNAP2,	*Solanum lycopersicon*	Promote	Transcription level	Ma et al., 2018
SlORE1S06, SlORE1S03, and SlORE1S02	*Solanum lycopersicon*	Promote	Transcription level	Lira et al., 2017
SiNAC1	*Setaria italica*	Promote	Transcription level	Ren et al., 2018
GmNAC065 and GmNAC085	*Glycine max*	Promote	Transcription level	Melo et al., 2018
GmNAC81	*Glycine max*	Promote	Transcription level	Pimenta et al., 2016
GhNAC12	*Gossypium hirsutum*	Promote	Transcription level	Zhao et al., 2016
AIF2	*Arabidopsis thaliana*	Delay	Transcription level	Kim et al., 2020
AP2	*Arabidopsis thaliana*	Delay	Transcription level	Balanza et al., 2018
bHLH03, bHLH13, bHLH14, and bHLH17	*Arabidopsis thaliana*	Delay	Transcription level	Qi et al., 2015
CRF1, CRF2, CRF3, CRF5, and CRF6	*Arabidopsis thaliana*	Promote	Transcription level	Raines et al., 2016
CBF2	*Arabidopsis thaliana*	Delay	Transcription level	Sharabi-Schwager et al., 2010
DEAR1	*Arabidopsis thaliana*	Promote	Transcription level	Tsutsui et al., 2009
FYF	*Arabidopsis thaliana*	Delay	Transcription level	Chen et al., 2011
IAA17	*Arabidopsis thaliana*	Promote	Transcription level	Shi et al., 2015
KHZ1 and KHZ2	*Arabidopsis thaliana*	Promote	Transcription level	Yan et al., 2017
MYC2, MYC3, and MYC4	*Arabidopsis thaliana*	Promote	Transcription level	Qi et al., 2015
MYC5	*Arabidopsis thaliana*	Promote	Transcription level	Song et al., 2017
JAZ7	*Arabidopsis thaliana*	Promote	Transcription level	Yu et al., 2016
MdbHLH3	*Malusdomestica*	Promote	Transcription level	Hu et al., 2020
AtMYBL	*Arabidopsis thaliana*	Promote	Transcription level	Zhang et al., 2011
MYB2	*Arabidopsis thaliana*	Promote	Transcription level	Buchanan-Wollaston et al., 2005
MYBR1	*Arabidopsis thaliana*	Delay	Transcription level	Jaradat et al., 2013
MYBH	*Arabidopsis thaliana*	Promote	Transcription level	Huang et al., 2015
ORE15	*Arabidopsis thaliana*	Delay	Transcription level	Kim J. H. et al., 2018
OsMYC2	*Oryza sativa*	Promote	Transcription level	Uji et al., 2017
OsMYB102	*Oryza sativa*	Delay	Transcription level	Piao et al., 2019
Rap2.4f	*Arabidopsis thaliana*	Promote	Transcription level	Xu et al., 2010
REVOLUTA	*Arabidopsis thaliana*	Promote	Transcription level	Xie Y. et al., 2014
RAV1	*Arabidopsis thaliana*	Promote	Transcription level	Woo et al., 2010
ScMYB2S1	*Sugarcane*	Promote	Transcription level	Guo X. et al., 2017
SlERF36	*Solanum lycopersicon*	Promote	Transcription level	Upadhyay et al., 2013
SUB1A	*Oryza sativa*	Delay	Transcription level	Fukao et al., 2012
SlFYFL	*Solanum lycopersicon*	Delay	Transcription level	Xie Q. et al., 2014
SlMBP11	*Solanum lycopersicon*	Delay	Transcription level	Guo X. et al., 2017
TCP2, TCP4, and TOP10	*Arabidopsis thaliana*	Promote	Transcription level	Schommer et al., 2008
CFM4	*Arabidopsis thaliana*	Promote	Post-transcriptional level	Lee et al., 2014
ERF4	*Arabidopsis thaliana*	Promote	Post-transcriptional level	Koyama et al., 2013; Riester et al., 2019
miR156	*Arabidopsis thaliana*	Delay	Post-transcriptional level	Wang, 2014
miR164	*Arabidopsis thaliana*	Delay	Post-transcriptional level	Kim et al., 2009
miR172	*Zea mays*	Delay	Post-transcriptional level	Wu et al., 2016
miR319	*Arabidopsis thaliana*	Promote	Post-transcriptional level	Schommer et al., 2008
miR840	*Arabidopsis thaliana*	Delay	Post-transcriptional level	Yujun et al., 2019
ONAC054	*Oryza sativa*	Promote	Post-transcriptional level	Sakuraba et al., 2020
PtRD26	*Populus*	Delay	Post-transcriptional level	Wang et al., 2021
SlymiR208	*Solanum lycopersicon*	Promote	Post-transcriptional level	Zhang Y. et al., 2020
ScMYB2	*Saccharum officinarum*	Promote	Post-transcriptional level	Guo X. et al., 2017

(Continued)

TABLE 1 | (Continued)

Gene	Species	Effects	Regulation	References
u11-48k	*Arabidopsis thaliana*	Promote	Post-transcriptional level	Xu et al., 2016
eIF5A	*Picrorhiza kurrooa*	Delay	Translation level	Parkash et al., 2014
LreEF1A4	*Petunia hybrida*	Delay	Translation level	Sun et al., 2020
SPL33	*Oryza sativa*	Delay	Translation level	Wang et al., 2017
AtSARK	*Arabidopsis thaliana*	Promote	Post-translational level	Xu et al., 2011
AtWAKL10	*Arabidopsis thaliana*	Delay	Post-translational level	Li L. et al., 2021
ATG4a/4b	*Arabidopsis thaliana*	Delay	Post-translational level	Yoshimoto et al., 2004
ATG9	*Arabidopsis thaliana*	Delay	Post-translational level	Hanaoka et al., 2002
ATG10	*Arabidopsis thaliana*	Delay	Post-translational level	Phillips et al., 2008
ATG18a	*Arabidopsis thaliana*	Delay	Post-translational level	Xiong et al., 2005
EDR1	*Arabidopsis thaliana*	Delay	Post-translational level	Frye et al., 2001
MKK4/5,MPK1/2	*Arabidopsis thaliana*	Promote	Post-translational level	Zhang Y. et al., 2020
MAPKKK18	*Arabidopsis thaliana*	Promote	Post-translational level	Matsuoka et al., 2015
MPK6/MKK9	*Arabidopsis thaliana*	Promote	Post-translational level	Zhou et al., 2009
GmSARK	*Glycine max*	Promote	Post-translational level	Li et al., 2006
OsMAPKKK1	*Oryza sativa*	Promote	Post-translational level	Wang et al., 2015
PUB12/PUB13	*Arabidopsis thaliana*	Delay	Post-translational level	Zhou et al., 2015
RPN10	*Arabidopsis thaliana*	Promote	Post-translational level	Lin et al., 2011
RPN5a	*Arabidopsis thaliana*	Promote	Post-translational level	Book et al., 2009
SAUR49	*Arabidopsis thaliana*	Promote	Post-translational level	Wen et al., 2020
SERK4	*Arabidopsis thaliana*	Delay	Post-translational level	Li et al., 2019
UPL5	*Arabidopsis thaliana*	Delay	Post-translational level	Zentgraf et al., 2010
UBP12/UBP13	*Arabidopsis thaliana*	Promote	Post-translational level	Park et al., 2019
UBA2	*Arabidopsis thaliana*	Promote	Post-transcriptional level	Kim et al., 2008

process (Robatzek and Somssich, 2001; Miao et al., 2004; Zentgraf et al., 2010; Zhou et al., 2011; Besseau et al., 2012; Jiang et al., 2014; Chen et al., 2017; Guo P. et al., 2017; Niu et al., 2020; Wang et al., 2020). Recently, WRKY46 was found to interact with Non-expressor of Pathogenesis-Related gene 1 (NPR1) and combined with the WRKY6 promoter to induce its expression in response to SA signals, thereby establishing an NPR1-WRKY46-WRKY6 signaling cascade to regulate leaf senescence (Zhang D. et al., 2021). Several WRKY TFs coordinate leaf growth and senescence in plants, including GhWRKY42 and GhWRKY91 in *Gossypium hirsutum*, BrWRKY6 in cabbage (Brassica rapa), CpWRKY71 in *Chimonanthus praecox*, OsWRKY93 in *Oryza sativa*, BnaWSR1 (WRKY regulating SA and ROS 1) and BnaWGR1 (WRKY generating ROS 1) in *Brassica napus* (Fan et al., 2018; Gu et al., 2018, 2019; Yang et al., 2018; Huang et al., 2019; Cui et al., 2020; Li Y. et al., 2021).

NAC family is one of the largest gene families in plants and plays a central role in regulating leaf senescence. NAC TFs function as positive regulators of leaf senescence, including ORE1/ANAC092, AtNAP/ANAC029, ORE1 SISTER1 (ORS1/ANAC059), ATAF2/ANAC081, ATAF1/ANAC002, ANAC019, AtNAC3/ANAC055, RESPONSIVE TO DESICCATION 26 (ATRD26/ANAC072), ANAC102, ANAC032, ANAC046, ANAC016, and NAC TRANSCRIPTION FACTOR-LIKE 9 (NTL9) or negative regulators such as JUNGBRUNNEN1 (JUB1/ANAC042), ANAC017, VND-INTERACTING1 (VNI1/ANAC082), VND-INTERACTING2 (VNI2/ANAC083), ANAC090, and ANAC075 (Guo and Gan, 2006; Yoon et al., 2008; Kim et al., 2009, 2013; Balazadeh et al., 2011; Nakashima et al., 2012; Wu et al., 2012; Hickman et al., 2013; Garapati et al., 2015; Lee et al., 2015; Takasaki et al., 2015; Kim H. J. et al., 2016, 2018; Li S. et al., 2016; Mahmood et al., 2016; Oda-Yamamizo et al., 2016; Woo et al., 2016; Nagahage et al., 2020; Kan et al., 2021). Future studies need to investigate whether there is communication between these positive and negative regulatory NAC-TFs, which will help to gain insight into the fine regulatory mechanisms of leaf senescence. On the one hand, to investigate whether there are direct interactions between these factors and whether they are synergistic or antagonistic to each other. On the other hand, ChIP-Seq data should be used to analyze whether their target genes overlap to develop a gene regulatory network of leaf senescence. Indeed, some studies have already started to address this aspect. For example, members of NAC-TFs and WRKY-TFs have been found to interact with each other to change the expression of downstream target genes, which in turn triggers leaf senescence (Kim et al., 2009; Zentgraf et al., 2010; Balazadeh et al., 2011; Besseau et al., 2012; Kim H. J. et al., 2016, 2018). Since the process of leaf senescence is accompanied by nutrient return, genes that regulate senescence are likely to regulate crop yield. In supporting this hypothesis, several NAC-TFs regulate crop yield by fine-tuning the initiation and progression of leaf senescence, such as NAM-B1 and TaNAC-S in wheat (Uauy et al., 2006; Zhao et al., 2015; Kang et al., 2019; Sakuraba et al., 2020; Yang et al., 2020; Yan et al., 2021), which provides a molecular strategy to improve crop yield or quality by finely regulating the leaf senescence process.

The basic helix-loop-helix (bHLH) family TFs also regulate leaf senescence. Members of bHLH subgroup IIIe factors, including myelocytomatosis protein 2 (MYC2), MYC3 and MYC4, antagonistically interact with the bHLH subgroup IIId factors bHLH03 (JAM3), bHLH13 (JAM2), bHLH14, and bHLH17 (JAM1), and mediate JA-induced leaf senescence by directly binding the promoter of *SAG29* in Arabidopsis (Qi et al., 2015; Goossens et al., 2017). In addition, MYC5 also positively regulates JA-induced leaf senescence (Song et al., 2017). Darkness induces the protein accumulation of JASMONATE ZIM-domain 7(JAZ7), which in turn inhibits dark-induced leaf senescence by suppressing MYC2 (Yu et al., 2016). In rice, OsMYC2 acts as a positive regulator of leaf senescence by regulating the transcript levels of *SAG*s (Uji et al., 2017). These findings suggest that MYC2 regulates leaf senescence via multiple signaling pathways. ACTIVATION-TAGGED BRI1 (BRASSINOSTEROID-INSENSITIVE1)-SUPPRESSOR1 (ATBS1)-INTERACTING FACTOR2 (AIF2) is a non-DNA-binding bHLH TF and delays dark or BR-induced leaf senescence (Kim et al., 2020). Phytochrome-interacting bHLH transcription factors (PIFs) such as PIF4 and PIF5 promote leaf senescence under natural or dark conditions in Arabidopsis (Sakuraba et al., 2014; Song et al., 2014; Li N. et al., 2021). MdbHLH3 regulates leaf senescence by promoting the expression of *dehydratase-enolase-phosphatase complex 1* (*MdDEP1*) in *Malus Domestica* (Hu et al., 2020). These results imply that bHLH TFs are involved in the regulation of leaf senescence in both annuals and perennial woody plants.

There is growing evidence that multiple TF families of genes are involved in the regulation of leaf senescence, including MYB-TFs such as MYB2 (Buchanan-Wollaston et al., 2005), MYB DOMAIN PROTEIN R1 (MYBR1) (Jaradat et al., 2013), MYB HYPOCOTYL ELONGATION-RELATED (MYBH) (Huang et al., 2015), AtMYBL (Zhang et al., 2011), OsMYB102 (Piao et al., 2019), ScMYB2S1 (Guo J. et al., 2017); PLANT A/T-RICH SEQUENCE- AND ZINC-BINDING PROTEIN (PLATZ) family transcription factor (ORE15) (Kim J. H. et al., 2018); AP2/ERF transcription factors such as CRF1/2/3/5/6 in Arabidopsis (Raines et al., 2016), SlERF36 in Tomato (Upadhyay et al., 2013), and SUBMERGENCE1A (SUB1A) in Rice (Fukao et al., 2012); AP2/DREB transcription factors (DEAR1 and Rap2.4f) (Tsutsui et al., 2009; Xu et al., 2010); CCCH zinc-finger family [K-homolog (KH) proteins, KHZ1 and KHZ2] (Yan et al., 2017); AUXIN RESISTANT 3 (AXR3)/INDOLE-3-ACETIC ACID INDUCIBLE 17 (IAA17), one member of Auxin response factors (ARF) family, is a positive regulator of natural leaf senescence (Shi et al., 2015); TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) family transcription factor (TCP2/4/10) (Schommer et al., 2008); Homeodomain-leucine zipper family (REVOLUTA) (Xie Y. et al., 2014); MADS box transcription factors such as FOREVER YOUNG FLOWER (FYF) in Arabidopsis and SlFYFL in Tomato (Chen et al., 2011; Xie Q. et al., 2014; Guo X. et al., 2017), as well as RAV family transcription factor (RAV1) (Woo et al., 2010).

A large number of studies have shown that TF plays a key regulatory role in leaf senescence, however, most of the studies have mainly focused on a few families, including the NAC or WRKY families, and more studies are needed in the future to analyze whether other family TFs are also involved in leaf senescence. A recent transcriptomic study revealed that 115 Sen-TFs from 31 families are involved in autumn leaf senescence in poplar (Wang et al., 2021), further supporting this suggestion.

Post-transcriptional Level

Post-transcriptional regulation, including RNA editing, polyadenylation, mRNA stability, and alternative splicing, is related to leaf senescence. Multiple organellar RNA editing factors 9 (MORF9), one of the core proteins of plant editosomes, are involved in the RNA editing in chloroplasts, and its mRNA level declined in senescent leaves (Tian et al., 2019). MicroRNA (miRNA) is involved in leaf senescence by regulating the expression of *SAG* genes. For example, miR156, miR164, miR172, and miR840 regulate leaf senescence by suppressing their target genes (Kim et al., 2009; Wu et al., 2016, 2020; Tian et al., 2019; Yujun et al., 2019; Roussin-Leveillee et al., 2020). In addition to miRNAs, circular RNA (circRNA), and long non-coding RNA (lncRNAs) participate in leaf senescence of rice by a competitive endogenous RNA (CeRNA) network (Huang et al., 2021a,b). Interestingly, EIN3 and clock-associated PSEUDO-RESPONSE REGULATOR 9 (PRR9) up-regulate the transcription level of *ORE1* by inhibiting the transcription of *miR164* (Li et al., 2013; Kim H. et al., 2018). miR398 participates in regulating leaf senescence by post-transcriptional regulation of *ASCORBATE PEROXIDASE 6* (*APX6*) (Chen et al., 2021). *SlymiR208* regulates leaf senescence by controlling the expression of isopentenyl transferases *SlIPT2* and *SlIPT4* (Zhang Y. et al., 2020).

Alternative splicing (AS) is widely used in RNA splicing and processing after gene transcription in higher eukaryotes, which can increase the diversity of transcriptome and proteome. AS events can be mainly classified into five categories: IR, skipping exon and mutually exclusive exons, as well as alternative 5'-splice sites and alternative 3'-splice sites. In animals, splicing factors control cellular senescence by regulating the splicing process of RNA precursors (Fregoso et al., 2013). In plants, AS acts as a regulatory mechanism of plant development or adaptation to environmental stress factors. RNA splicing factor RNA-BINDING PROTEIN 25 (RBM25) responds to ABA stress in *Arabidopsis* (Zhan et al., 2015). Loss-of-function of *CRM FAMILY MEMBER SUBFAMILY 4* (*CFM4*) leads to abnormal rRNA processing during chloroplast RNA splicing, and exhibited plant growth retardation and delayed senescence (Lee et al., 2014). An interesting discovery shows that the differential expression of sugarcane MYB TF ScMYB2 alternative splicing transcripts may be an important post-transcriptional regulatory mechanism for controlling drought stress and leaf senescence (Guo J. et al., 2017).

The splicing mechanism occurs in the spliceosome, which is composed of five small nuclear RNAs (snRNAs) and a series of related protein factors. The spliceosome can recognize the splice site of the precursor RNA and catalyze the splicing reaction. There are major spliceosomes (U2) and minor spliceosomes (U12) that support splicing functions (Sharp, 2005). Although the splicing efficiency of the U12-type spliceosome is relatively lower,

splicing errors will affect the normal growth and development of plants. The U12-type intron-specific small spliceosome mainly removes the small U12 intron from the precursor mRNA. Mutation of *u11-48k* causes defects in growth and development, such as short plant size, increased lotus-like leaves, and delayed senescence (Xu et al., 2016), indicating that the regulation of the RNA splicing process has a potentially important effect on plant leaf senescence. ETHYLENE RESPONSE FACTOR4 (ERF4) has two different isoforms, ERF4-R and ERF4-A, produced by alternative polyadenylation of its pre-mRNA. ERF4-R, contains an ERF-associated amphiphilic repression (EAR) motif and acts as a repressor, whereas the other form, ERF4-A, is lacking this motif and acts as an activator. *ERF4-R* and *ERF4-A* can directly bind to the promoter of *CATALASE3* (*CAT3*) but have antagonistic effects on gene expression. The ratio of *ERF4-A* to *ERF4-R* mRNA changed as the plant ages and caused a complex age-dependent regulation of CAT3 activity. Interestingly, overexpression of *ERF4-R* but not of *ERF4-A* led to accelerated senescence (Koyama et al., 2013; Riester et al., 2019). ONAC054 was shown to participate in ABA-induced leaf senescence by directly activating *OsABI5* in rice (Sakuraba et al., 2020). Interestingly, the *ONAC054* transcript (*ONAC054*α) has an alternatively spliced form, *ONAC054*β, encoding a small truncated protein. Overexpression of *ONAC054*α or *ONAC054*β promotes leaf senescence (Sakuraba et al., 2020). A recent study reported that an alternative splicing event retaining the first intron of the *PtRD26* pre-mRNA occurred in a senescence-associated manner in poplar. The intron retention (IR) event in *PtRD26* led to an alternative splicing variant, PtRD26^{IR}, which encodes a truncated protein. PtRD26^{IR} forms heterodimers with multiple hub Sen-NAC TFs, including PtNAC039, PtNAC055, PtNAC076, PtNAC086, PtNAC099, and PtNAC109, represses their DNA binding activity to target genes, and delays age-, dark,- and PtRD26-induced leaf senescence in poplar, tobacco, and *Arabidopsis*. PtRD26 regulates Sen-NAC TFs by directly binding their promoters or indirectly through protein-protein interactions using its splicing variant, PtRD26^{IR}, thereby forming a multiply-interlocked feed-forward loop to finely tune the leaf senescence process. Functional analysis of senescence-associated splicing factors (SF) revealed that *PtU2A2A*, *PtU2A2B-1*, or *PtU2A2B-2* (U2 auxiliary factor large subunit A or B) are involved in AS of PtRD26^{IR}. Silencing separately or simultaneously of these SFs significantly decreased the transcript levels of *PtRD26*^{IR} and accelerated leaf senescence. Based on these findings, it is found that the products of AS have different functions and regulate plant development such as plant senescence through different mechanisms. With the application of multi-omics technology, more AS events will be found to be involved in the regulation of leaf senescence, which will Further deepen the mechanistic understanding of plant aging.

Translation Level

Senescence is a long-term state of cell cycle arrest arising from cells that have suffered sublethal damage. Although senescent cells no longer replicate, they remain metabolically active and further develop a distinct and stable phenotype not seen in proliferating cells (Guo and Gan, 2005; Lim et al., 2007). On the one hand, along with leaf senescence, a large number of proteins are degraded and translation efficiency decreases; on the other hand, senescence-specific regulatory factors are synthesized to inhibit or retard the leaf senescence process (Guo and Gan, 2005; Lim et al., 2007). Thus, translation in senescent cells paradoxically includes a general inhibition of translation triggered by numerous stresses and a selective increase in translation of specific proteins, including SAG protein.

Mutation of *ORE4*, which encodes the plastid ribosomal small subunit protein 17 that is a component of the plastid ribosome, reduces the translation rate in the chloroplast and thus extends leaf longevity in *Arabidopsis* (Woo et al., 2002), suggesting a possible link between decreased metabolism and extended longevity of the leaves. Translation initiation, the first step in the protein synthesis process, is the main regulatory step controlling translation and involves a large number of translation initiation factors. Studies in plants have revealed that these translation initiation factors affect various aspects of plant growth and development, in addition to their role in protein synthesis (Wang et al., 2001). Mutation of *EUKARYOTIC ELONGATION FACTOR 5A* (*eIF5A*) significantly inhibits plant nutrition and reproductive growth and delays leaf senescence in *Arabidopsis* and Picrorhiza (*Picrorhiza kurrooa Royle ex Benth.*) (Reviron et al., 1992; Parkash et al., 2014). Translation initiation factor eIF3h is involved in the signal activation and restart of rapamycin (TOR) and affects the growth and development of plants (Schepetilnikov et al., 2013). In addition, eukaryotic translation elongation factors (eEF) also involved leaf senescence. For instance, mutation of *Spotted Leaf 33* (*spl33*), encoding a eEF1 alpha (eEF1A)-like protein, induces early leaf senescence (Wang et al., 2017). Ectopic expression of *Lilium regales Eukaryotic translation elongation factor 1 alpha 4* (*LreEF1A4*), encoding the α subunit of elongation factor 1 from a *Lilium regale* cucumber mosaic virus (CMV), delayed leaf and flower senescence in petunia (*Petunia hybrida*) (Sun et al., 2020). Interestingly, two subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key enzyme that determines the rate of carbon assimilation in photosynthesis, is controllable at the translation level, and affect plant growth and development, including the leaf senescence process (Suzuki and Makino, 2013; Woo et al., 2013).

Post-translational Level

Post-translational modifications (PTM), including methylation, acetylation, phosphorylation, ubiquitination, and deubiquitination affect the structure and function of proteins. Previous studies found that the PTM of a large number of SAG proteins changed with leaf senescence (Wang and Schippers, 2019). This implies a close relationship between leaf senescence and PTM, but the causal relationship is not clear.

Transcriptomics analysis reveals that a large number of SAGs are involved in PTM, such as receptor-like kinase (RLK) and mitogen-activated protein kinase (MAPK) (Ahmad and Guo, 2019). RLK is an ideal candidate for senescence-inducing signal receptors, which often have an N-terminal extracellular binding domain for ligand binding, a transmembrane domain spanning the plasma membrane, and a cytoplasmic kinase domain (Shiu and Bleecker, 2001; Gish and Clark, 2011). The

largest subfamily of RLK is the leucine-rich repeat receptor-like protein kinase (LRR-RLK), containing more than 200 members, and lots of them are involved in the regulation of leaf senescence (Shiu et al., 2004). GmSARK (*Glycine max* Senescence-Associated Receptor-like Kinase), a senescence-associated LRR-RLK isolated from soybean (*Glycine max*) and its homolog AtSARK in *Arabidopsis* are positive regulators of leaf senescence (Li et al., 2006; Xu et al., 2011). SARK-mediated signaling pathway positively regulates leaf senescence through suppressing SMALL AUXIN-UP RNA 49 (SAUR49), a negative regulator of leaf senescence, and activating SENESCENCE-SUPPRESSED PROTEIN PHOSPHATASE (SSPP), an accelerator of leaf senescence (Xiao et al., 2015; Wen et al., 2020). In contrast, the somatic embryogenesis receptor-like kinase 4 (SERK4) and the cell wall-associated kinase 10 (AtWAKL10) act as the negative regulators of leaf senescence (Li et al., 2019; Li L. et al., 2021). Interestingly, a common receptor can work with multiple receptors in different signaling pathways. AtSARK and SERK4 may be part of the receptor complex that regulates plant aging by acting with other LRR-RLKs (Brandt and Hothorn, 2016; Cui et al., 2018).

The mitogen-activated protein kinase cascade MAPKKK-MAPKK-MAPK is one of the most important signal transduction pathways in plants and animals. Recently, MAP KINASE 4/5 (MKK4/5)-MITOGEN-ACTIVATED PROTEIN KINASE 1/2 (MPK1/2), MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 18 (MAPKKK18), and OsMAPKKK1 have been found to be the positive regulators of leaf senescence (Matsuoka et al., 2015; Wang et al., 2015; Zhang J. et al., 2020). By contrast, Enhanced Disease Resistance 1 (EDR1), a MAPKK, functions as a negative regulator by coordinating biotic stress response and ethylene-induced senescence (Frye et al., 2001; Tang and Innes, 2002). MKK9 phosphorylates the target MPK6, which stabilizes the leaf senescence transcription factor EIN3 by promoting the cleavage and nuclear translocation of ORE3/EIN2 (Zhou et al., 2009; Zhang Y. et al., 2016). These findings suggest that RLKs and MAPKs regulate leaf senescence by affecting the phosphorylation status of target proteins.

The leaf senescence process is accompanied by protein degradation. The main protein degradation pathways are autophagy and the ubiquitin-proteasome system (UPS), which precisely regulate the turnover of organelles and the degradation of abnormal proteins and maintain protein homeostasis. Autophagy and protein ubiquitination are synergistic in the cell. Ubiquitination acts as a signal to induce organelles to target autophagy. Mitophagy and chloroplast protein degradation is the result of the synergistic effect of ubiquitination and autophagy (Geisler et al., 2010; Kikuchi et al., 2020). Interestingly, autophagy seems to prevent aging, whereas the proteasome acts as a positive regulator of aging (Wang and Schippers, 2019). Chaperone-mediated autophagy is one of the main types of autophagy in cells, with high selectivity. Autophagy-related genes (ATG) involved in autophagy are up-regulated with the occurrence of plant senescence (Masclaux-Daubresse et al., 2014). Mutation of several ATG genes, including ATG4a/4b, ATG9, ATG19, and ATG18a, promotes leaf senescence under nitrogen-starvation conditions (Hanaoka et al., 2002; Yoshimoto et al., 2004; Xiong et al., 2005; Phillips et al., 2008; Wang and Schippers, 2019). Although most studies support the role of autophagy in delaying aging, ATG8 promotes senescence by interacting with the ABNORMAL SHOOT3 (ABS3). This non-autophagic ATG8-ABS3 pathway interacts with the classic autophagy pathway to balance aging and survival (Jia et al., 2019). Therefore, the components of autophagy may have a dual role in the initiation and progression of senescence. 26S proteasome is mainly responsible for degrading ubiquitinated proteins. The recognition of ubiquitinated substrates in the process of ubiquitin/proteasome-mediated proteolysis (UPP) is directly mediated by the proteasome subunits RPN10 (REGULATORY PARTICLE NON-ATPase 10) and RPN13. The loss of the potential UPP ubiquitin receptor *RPN10* significantly delays senescence (Lin et al., 2011), and overexpression of *RPN5a* leads to premature senescence (Book et al., 2009). In contrast to the overall up-regulation of ATG genes, transcript levels of only a small part of the proteasome subunit genes were increased during leaf senescence (Guo and Gan, 2012). In the senescent leaf of rape and barley (*Hordeum vulgare* L.), the proteasome is very active (Poret et al., 2016; Velasco-Arroyo et al., 2016). Interestingly, an application of protease inhibitor delays the onset of senescence symptoms (Pak and van Doorn, 2005). Taken together, these observations imply that autophagy and proteasome seem to have different effects on the onset of senescence, and they coordinately regulate the progression of leaf senescence.

One of the well-characterized PTMs involved in the regulation of leaf senescence is ubiquitination/deubiquitination modification. Protein ubiquitination requires the synergy of ubiquitin activation (E1), ubiquitin-binding (E2), and ubiquitin ligase (E3). Members of E2 and E3 have been found to be involved in the regulation of leaf senescence (Shu and Yang, 2017; Park et al., 2018). Among them, RING-type E3 and U-box-type E3 ligases have been shown to act as regulators of leaf senescence by mediating ABA signaling. For example, PLANT U-box (PUB) E3 ubiquitin ligase PUB12 and PUB13 ubiquitinated FLS2 (FLAGELLIN-SENSITIVE 2) for protein degradation, thereby down-regulating flagellin signaling and negatively regulating stress-induced leaf senescence (Zhou et al., 2015). In addition, HECT-type ubiquitin E3 ligase (UPL1-UPL7) plays a critical role in cell death and leaf senescence (Lan and Miao, 2019). Mutation of *UBIQUITIN PROTEIN LIGASE 5* (*UPL5*) leads to the accumulation of *WRKY53* and induces early leaf senescence (Zentgraf et al., 2010). Ubiquitin-specific protease (UBP1)-associated protein 2a (UBA2a), UBA2b, and UBA2c positive regulators of leaf senescence (Kim et al., 2008). Likewise, the potato (*Solanum tuberosum*) RNA-binding protein StUBA2a/b is homologous to *Arabidopsis* UBA2s. Constitutive overexpression of *StUBA2a/b* increases the expression of the *SAG13* gene, pathogen-related genes (PR), and autophagy-related genes, and promotes leaf senescence in *Arabidopsis* (Na et al., 2015). The process of protein ubiquitination is reversible, and deubiquitinating enzymes (DUBs) can remove mono-ubiquitin molecules or polyubiquitin chains on proteins. UBP is the largest DUB subfamily, and members of the UBP family are involved in a variety of physiological processes, including leaf senescence (Zhou et al., 2017). Out of them,

UBIQUITIN-SPECIFIC PROTEASE 12 (UBP12) and UBP13 are involved in the regulation of circadian clock and flowering (Cui et al., 2013), and accelerate nitrogen starvation-induced leaf senescence by counteracting the effect of E3 ligase NLA (Nitrogen Ubiquitin-Protein Ligases DNA) to maintain the homeostasis of ORE1 (Park et al., 2019).

CONCLUSIONS AND PERSPECTIVES

Leaf senescence is a highly complex process of orderly degradation of cell structure and is controlled by multiple layers regulatory network (**Figure 1**), in which different regulatory factors at different levels may interact to fine-tune the initiation and progression of leaf senescence (**Table 1**). Although regulation is artificially divided into multiple levels (Woo et al., 2013), leaf senescence is a highly dynamic regulatory process (Woo et al., 2019), and there is no single way to regulate it. For example, changes in chromatin structure affect gene expression, protein translation, and thus the function of transcription factors, which in turn cause changes in the senescence process of plant leaves. Moreover, the regulation of leaf senescence involves not only the interactions between proteins, proteins, and DNA, but also the exchange of information between cells and organelles, thus synergistically regulating the initiation of leaf senescence, which guarantees the return of nutrients and the survival of plants. Therefore, we should combine genome, transcriptome, proteome, metabolome, and the latest translation comics data to discuss the general mechanism of regulate senescence and understand how senescence and death are systemically integrated within the entire plant (Kim J. et al., 2016).

With the aid of forwarding or reversing genetics strategies and the development of multi-functional CRISPR genome editing technology, a large number of senescence-related mutants will be generated. For example, quintuple mutants of *oss40s-cr* generated using CRISPR technology displays stay-green phenotypes (Habiba et al., 2021), which will further deepen our understanding of leaf senescence. The model plant Arabidopsis has played an important role in revealing the molecular or genetic regulation mechanisms of plant senescence, but we still know little about leaf senescence and do not fully understand the biological significance of senescence (Lim et al., 2007). The relatively short life cycle of Arabidopsis has limitations for our understanding of plant aging. Along with the genomic information revealed for a variety of plants, it provides the possibility to systematically study plant senescence by comparative genomics.

It's unclear how these transcription factors regulate, such as the WRKY family and NAC family, and epigenetic factors co-regulate the senescence process of plants. The function of hormone signaling on leaf senescence has been widely recognized (Hu et al., 2017). It is necessary to further explore how plant signals and environmental signals are integrated into the hormone signaling pathway, and how post-translational modifications such as phosphorylation and ubiquitination are passed through transcription factors, kinases, and protease, finely control these signals to regulate gene expression and protein turnover during leaf senescence. The senescence symptoms of leaf senescence have always been detected at the organ level. However, in senescent leaves, leaf cells are usually at different developmental ages or senescence stages, which makes it impossible to better understand the biological process of leaf senescence. Fortunately, the application of single-cell sequencing technology may offer the possibility to resolve the cytological basis of leaf senescence.

In addition to the loss- or gain-of-function of mutants, ecotypes of various species will greatly contribute to the understanding of the molecular mechanisms underlying leaf senescence. Through analysis of naturally occurring DNA methylation variation regions (NMRs) between Col-0 and C24 accessions of *Arabidopsis thaliana*, a retrotransposon named NMR19-4 (naturally occurring DNA methylation variation region 19) was identified to be involved in the regulation of leaf senescence (He et al., 2018). NMR19-4 is an environmentally associated epiallele that controls leaf senescence by regulating the expression of PHEOPHYTIN PHEOPHORBIDE HYDROLASE (PPH), which is involved in chlorophyll breakdown (Schelbert et al., 2009; He et al., 2018). By mapping the quantitative trait locus (QTL) of leaf senescence between the Col-0 and Ct-1 accessions of *Arabidopsis thaliana,* ACCELERATED CELL DEATH 6 (ACD6) was identified as the causal gene (Jasinski et al., 2020). Using two rice subspecies indica and japonica, variations were found in the promoter regions of the Stay-Green (OsSGR) gene encoding a chlorophyll-degrading enzyme. This promoter variations trigger higher and earlier induction of OsSGR, which in turn accelerates leaf senescence in indica (Shin et al., 2020).

AUTHOR CONTRIBUTIONS

ZL conceived the project and designed the manuscript. HG and XX designed part of the manuscript. Y-MZ collected the data and organized figure. PG organized table. All authors have read and agreed to the published version of the manuscript.

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The Transcriptional Corepressor HOS15 Mediates Dark-Induced Leaf Senescence in Arabidopsis

*Shah Zareen[1†], Akhtar Ali[1,2†], Chae Jin Lim[1†], Haris Ali Khan[1], Junghoon Park[1], Zheng-Yi Xu[3] and Dae-Jin Yun[1,3]**

[1]Department of Biomedical Science and Engineering, Konkuk University, Seoul, South Korea, [2]Center of Plant Systems Biology and Biotechnology, Plovdiv, Bulgaria, [3]Key Laboratory of Molecular Epigenetics of the Ministry of Education (MOE), Northeast Normal University, Changchun, China

Multiple endogenous and environmental signals regulate the intricate and highly complex processes driving leaf senescence in plants. A number of genes have been identified in a variety of plant species, including Arabidopsis, which influence leaf senescence. Previously, we have shown that HOS15 is a multifunctional protein that regulates several physiological processes, including plant growth and development under adverse environmental conditions. HOS15 has also been reported to form a chromatin remodeling complex with PWR and HDA9 and to regulate the chromatin structure of numerous genes. However, unlike PWR and HDA9, the involvement of HOS15 in leaf senescence is yet to be identified. Here, we report that HOS15, together with PWR and HDA9, promotes leaf senescence *via* transcriptional regulation of *SAG12/29,* senescence marker genes, and *CAB1/RCBS1A,* photosynthesis-related genes. The expression of *ORE1, SAG12*, and *SAG29* was downregulated in *hos15-2* plants, whereas the expression of photosynthesis-related genes, *CAB1* and *RCBS1A*, was upregulated. HOS15 also promoted senescence through dark stress, as its mutation led to a much greener phenotype than that of the WT. Phenotypes of double and triple mutants of HOS15 with PWR and HDA9 produced phenotypes similar to those of a single *hos15-2*. In line with this observation, the expression levels of *NPX1*, *APG9*, and *WRKY57* were significantly elevated in *hos15-2* and *hos15/pwr, hos15/hda9, and hos15/pwr/hda9* mutants compared to those in the WT. Surprisingly, the total H3 acetylation level decreased in age-dependent manner and under dark stress in WT; however, it remained the same in *hos15-2* plants regardless of dark stress, suggesting that dark-induced deacetylation requires functional HOS15. More interestingly, the promoters of *APG9*, *NPX1*, and *WRKY57* were hyperacetylated in *hos15-2* plants compared to those in WT plants. Our data reveal that HOS15 acts as a positive regulator and works in the same repressor complex with PWR and HDA9 to promote leaf senescence through aging and dark stress by repressing NPX1, APG9, and WRKY57 acetylation.

Keywords: HOS15, leaf senescence, developmental aging, dark stress, chromatin remodeling

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***Correspondence:**
Dae-Jin Yun
djyun@konkuk.ac.kr

[†]These authors have contributed equally to this work

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INTRODUCTION

Leaf senescence is a programmed degeneration process that constitutes the final step of leaf development. Senescence is an organized process regulated by chlorophyll degradation, photosynthesis decline, lipid peroxidation, and protein deprivation (Smart, 1994). The initiation of leaf senescence is a developmentally programmed and apoptotic process that can be controlled through diverse signals, such as environmental factors (including light, nutrients, temperature, and osmotic stress), pathogen attack, and phytohormones (Lim et al., 2007a; Guo and Gan, 2012; Li et al., 2012). To date, several senescence-related mutants and a variety of senescence-associated genes (SAGs), which accumulate during leaf senescence, have been isolated and characterized (Buchanan-Wollaston et al., 2003; Lim et al., 2007a; Li et al., 2012). The expression levels of SAGs increase with leaf aging, while two photosynthetic genes, CHLOROPHYLL A/B BINDING PROTEIN 1 (CAB1) and RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A (RBCS1A), have been shown to be downregulated upon aging (Li et al., 2013). In addition, several NAC TFs have been identified in Arabidopsis and crop plants, which play important roles in senescence (Kim et al., 2016; Li et al., 2018). In particular, ANAC016, ANAC002/ATAF1, ANAC029/NAC-LIKE, ANAC019, ANAC032, ACTIVATED BY AP3/PI (NAP), ANAC092/ORESARA1 (ORE1), ANAC046, ANAC055, ANAC057/ORE1 SISTER 1 (ORS1), and ANAC072 promote senescence; ANAC083/VND-INTERACTING2 (VNI2) and ANAC042/JUNGBRUNNEN1 (JUB1) hinder senescence in Arabidopsis (Garapati et al., 2015; Takasaki et al., 2015; Kim et al., 2016; Oda-Yamamizo et al., 2016). The expression levels of NAC genes significantly increase during natural senescence (NS) and artificially induced senescence, such as dark-induced senescence (DIS; Kim et al., 2009, 2013; Sakuraba et al., 2015b). To explore the similarities and variabilities in gene expression levels during senescence and to measure the induction of SAGs by stress, is a major subject of plant researchers (Becker and Apel, 1993; Oh et al., 1996; Chung et al., 1997; Park et al., 1998; Weaver et al., 1998).

High Expression of Osmotically Responsive Genes 15 (HOS15), a WD40 repeat protein, has multiple molecular functions, including the regulation of plant growth and development, cold stress signaling, flowering time determination, abscisic acid (ABA) signaling, response to drought stress, and pathogen response (Zhu et al., 2008; Park et al., 2018a; Ali et al., 2019; Mayer et al., 2019; Park et al., 2019; Shen et al., 2020). During cold stress, HOS15 interacts with and promotes proteasomal degradation of histone deacetylase 2C (HD2C). In addition, it encourages histone 3 (H3) acetylation and keeps "open" the chromatin of cold-responsive (COR) genes and facilitates the recruitment of CBF TFs to the promoter of COR genes for cold stress tolerance (Park et al., 2018a). HOS15 also forms complexes with LUX, ELF3 (evening complex), and HDA9, which bind to the GI promoter and repress the transition to flowering (Park et al., 2019). Furthermore, HOS15 interacts with and degrades OST1, thereby regulating the desensitization of the ABA signaling pathway (Ali et al., 2019). In line with these reports, we have also shown that HOS15 interacts with the SCF-CUL4-E3 ligase complex to repress the plant immune system by negatively regulating NPR1, a pathogen-responsive positive regulator (Shen et al., 2020). Despite all these multiple functions, the role of HOS15 in senescence remains unknown.

In the present study, we report that HOS15 promotes leaf senescence in response to aging and dark stress. Phenotypically, and compared to wild-type (WT) plants, *hos15-2* mutants showed a dramatically late senescence phenotype. While *hos15-2* plants accumulated higher chlorophyll content as well as SAGs were also upregulated in loss-of-function HOS15 mutant plants in relation to WT plants. Moreover, transcript levels of NPX1, APG9, and WRKY57 were upregulated in *hos15-2* compared to WT. Interestingly, compared to WT, the acetylation status of total H3, AcK9, and AcK was higher in *hos15-2*, *pwr*, and *hda9* mutants. Furthermore, we also found a dark impede H3 acetylation level in WT compared *to hos15-2* plants, while the acetylation status of APG9, WRKY57, and NPX1 promoters was also higher in *hos15-2* plants than in WT plants. All these results indicate that HOS15 works in the same complex of PWR and HDA9 to regulate aging and dark-induced leaf senescence through the regulation of the same group of genes.

MATERIALS AND METHODS

Plant Materials

In the present study, the *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the WT. All the seeds used in the present study were from selected lines, such as Col-0 (WT), *hos15-2*, CL-1, CL-2, *pwr-2,* and *hda9-1*, as described in our previously published research articles (Ali et al., 2019; Baek et al., 2020; Khan et al., 2020). Seeds of the WT, complemented lines, and mutants were surface-sterilized in a solution containing 2% sodium hypochlorite solution (Yakuri Pure Chemicals, Kyoto, Japan) for 5 min and rinsed five times with sterilized water. After stratification for 3 days at 4°C in the dark, sterilized seeds were germinated on full-strength MS medium containing .25% phytagel and 2% sucrose. Ten-day-old seedlings were transferred to the soil under control conditions.

Growth Conditions

Plants were grown at 23°C under long-day conditions (16-h light/8-h dark photoperiod), under cool white, fluorescent light at a rate of 80–100 $\mu mol\, m^{-2} s^{-1}$ in a completely controlled culture room at Konkuk University, Seoul, South Korea. The green rosette and cauline leaves of the selected lines were detached from 4-week-old plants and were then sampled for age-wise leaf senescence phenotype.

Dark Treatment

For the dark treatment, 4-week-old WT, *hos15-2*, CL-1, and CL-2 plants were exposed to dark stress. However, in terms of leaves, the 1st and 2nd cauline leaves of each ecotype were detached and exposed to 4 days of dark stress. Photographs were taken before and after the dark stress treatment. RNA

was extracted from the same leaves, and cDNA was synthesized. Transcript levels were quantified through quantitative real-time PCR (qRT-PCR). In case of nuclear protein extraction to evaluate the histone acetylation status, 12-day-old seedlings of WT and *hos15-2* plants were covered in aluminum foil for 4 days. Nuclear proteins were extracted from stressed and unstressed seedlings using a nuclear protein extraction kit.

RNA Extraction and qRT-PCR Analysis

For the reverse transcription reactions (PCR), total mRNA (5 μg) was extracted from plants (harvested at different time points for each experiment) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNA was then treated with DNase-1 free Kit (Sigma, St. Louis, MO, United States), and cDNA was synthesized reverse transcription of total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, United States) with oligo $(dT)_{12}$ primer, according to the manufacturer's instructions. Quantitative PCR was performed using the SYBR Green PCR Master Mix kit (Bio-Rad, Hercules, CA, United States) according to instructions and using the CFX96 or CFX384 Real-time PCR detection system (Bio-Rad). The PCR mixture (20 μl) comprised 2 μl of first-strand cDNA template, 10 μl of LaboPass™ SYBR Green Q Master (CMQS1000), COSMO GENETECH, South Korea,[1] and .5 μm of forward and reverse primers for each gene. Three biological replicates were used for each genotype. The relative expression levels were calculated using the comparative cycle threshold method. The sequences of the primers used for the qRT-PCR are listed in **Supplementary Table 1**. Throughout the study, *ACTIN2* (*ACT2*) was used as the reference to determine relative normalized expression levels during qRT-PCR.

Chlorophyll Quantification

To measure the total chlorophyll content, frozen leaf tissue was homogenized with zirconia beads, and the pigment was extracted from the leaf homogenate with 80% frozen acetone. The total chlorophyll concentration was determined spectrophotometrically using Biomate 3 (Thermo Electron Corporation, United States), and the optical density (OD) was measured at 663 nm and 645 nm against an 80% acetone blank. The total chlorophyll content was determined using the following equation:

$$\text{Total chlorophyll}\left(\text{mg}/\text{g}\right) = \left[20.2^{*}\text{OD}_{645} + 8.02^{*}\text{OD}_{663}\right] \times \text{V}/1000 \times \text{W}$$

V = final volume and W = weight of a sample.

Nuclear Protein Extraction

Tissue samples (.5 g) of 3-week-old seedlings from selected genotypes were sampled in liquid nitrogen and ground manually. The CelLytic PN isolation/extraction Kit (Sigma) was used for nuclear protein extraction as previously described by Wang et al. (2011a).

Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation (ChIP) and ChIP-qRT-PCR were performed according to a previously reported method (Saleh et al., 2008). To fix the chromatin structure, 2-week-old Arabidopsis seedlings were treated with 1% formaldehyde for 15 min and then treated with .1 M glycine for 5 min to stop the cross-linking reaction. The plant tissue was ground with liquid nitrogen, washed with water, and the nuclei were extracted. Nuclear proteins were extracted and sonicated with a Bioruptor (BMS) to fragment the chromosomal DNA. Immunoprecipitation was conducted using the respective antibody, with salmon sperm carrier DNA and Protein-A agarose (Upstate Biotechnology).

Genetic Crosses

Genetic crosses were performed by transferring mature anthers from the donor to the stigmas of hand-emasculated female recipients.

RESULTS

HOS15 Positively Regulates Leaf Senescence

HOS15 is one of the 85 WD40 repeat proteins in Arabidopsis that function as substrate receptors for the DDB1-CUL4 E3 ligase complex (Lee et al., 2008). Previously, we reported that HOS15 is a multifunctional protein that regulates several physiological processes, including plant development and stress response (Ali and Yun, 2020). However, the involvement of HOS15 in the regulation of leaf senescence is yet to be explored. To investigate the role of HOS15 in leaf senescence, seeds of WT and loss-of-function HOS15 mutant (*hos15-2*) plants were germinated on Murashige and Skoog (MS) plates for 10 days and then transferred to soil. After 40 days of germination, we observed that *hos15-2* mutant plants showed a late senescence phenotype compared to WT and the two complementation lines (**Figure 1A**; **Supplementary Figure 1**). As leaf senescence has been considered the final stage of development from maturity to degeneration in the life history of plant leaves (Lim and Hong, 2007), we compared the rosette leaves (3rd to 12th leaves) after 40 days of germination of WT and *hos15-2*. We observed that *hos15-2* rosette leaves were greener than those of WT and the two complementation lines (**Figure 1B**; **Supplementary Figure 1B**). These results suggest that HOS15 promotes senescence in Arabidopsis. SAGs have been used as senescence markers because their transcript levels upregulate in an age-dependent (senescence) manner (Li et al., 2013; Sakuraba et al., 2014; Zhang et al., 2015; Ren et al., 2017). The transcript level of *SAG12,* a marker gene, was dramatically reduced in *hos15-2* plants compared to WT plants (**Figure 1C**; **Supplementary Figure 2**). Interestingly, *HOS15* was also induced transcriptionally in an age-dependent manner, suggesting the involvement of HOS15 in senescence

[1]http://www.cosmogenetech.com/co.kr

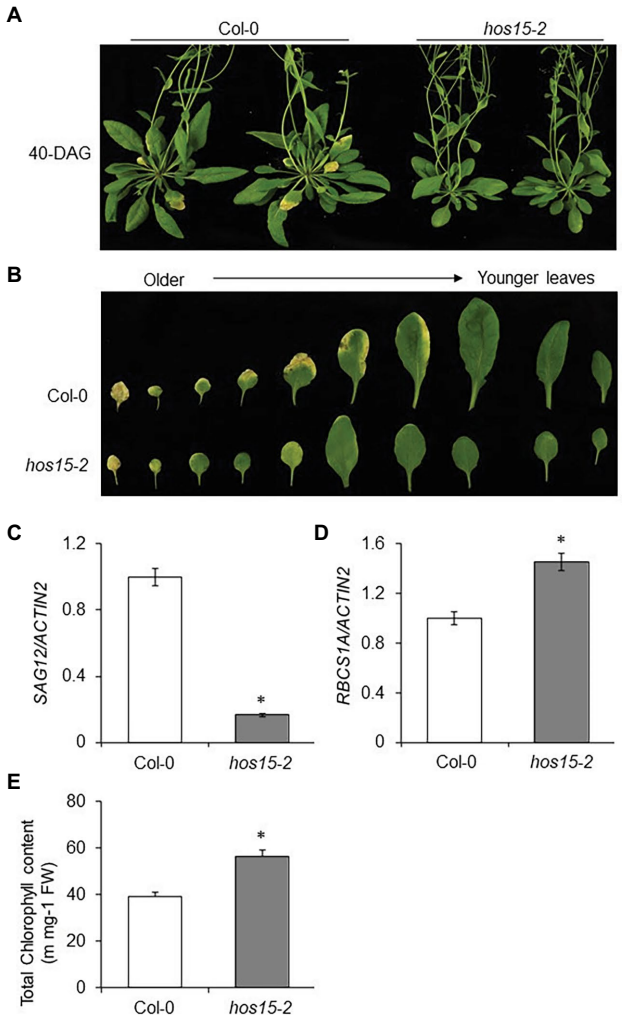

FIGURE 1 | The Arabidopsis *hos15-2* mutant shows late senescence phenotype. **(A)** Comparative phenotypic analysis of Col-0 (WT) and *hos15-2* plants after 40 days of germination. Seeds of Col-0 and *hos15-2* were sterilized and germinated on MS medium for 10 days and then transferred to soil. Photographs were taken after 40 days of germination (DAG). **(B)** Phenotypes of rosette leaves of Col-0 and *hos15-2* plants (3rd to 12th leaves) according to the sorted leaf age of 40 DAG. **(C,D)** Expression of *SAG12*, a senescence marker gene, and *RCBS1A*, a photosynthesis-related gene in 3rd and 4th leaves of Col-0 and *hos15-2* plants. Total RNA was extracted, and qRT-PCR analysis was performed. *ACTIN2* was used as the internal control. Error bars show SD. Differences were determined *via* Student's *t*-test (significance: $*p < .05$). **(E)** Leaves of the 4-week-old Col-0 and *hos15-2* plants (3rd and 4th rosette) were used for total chlorophyll content measurement. Error bars show SD. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$).

(**Supplementary Figure 2**). The transcript level of *RBCS1A* (small subunit of Rubisco 1A), a photosynthesis-related gene, was upregulated in *hos15-2* compared with WT (**Figure 1D**). In line with the RBCS1A transcript level, the total chlorophyll content also accumulated abundantly in *hos15-2* compared with WT plants (**Figure 1E**). Taken together, these results demonstrated that HOS15 acts as a positive regulator of leaf senescence.

HOS15 Regulates Senescence-Associated and Photosynthesis-Related Genes Differentially in an Age-Dependent Manner

Leaf senescence initiation is a naturally occurring complex process that starts with the upregulation of SAGs and repression of senescence downregulated genes (SDGs; Gepstein et al., 2003; Breeze et al., 2011; Brusslan et al., 2015). A decade ago, Kim et al. (2009) reported that the NAC-type TF family, particularly ORE1/NAC2, promote leaf senescence in an age-dependent manner. In contrast, photosynthesis-related genes, such as *RBCS1A* and *CAB1,* downregulate transcriptionally in an age-dependent manner (Bate et al., 1991; Weaver et al., 1997). To investigate whether HOS15 participates in senescence by regulating both SAGs and photosynthesis-related genes in an age-dependent manner, we analyzed the expression of SAGs in loss-of-function HOS15 mutant and WT plants. In *hos15-2, SAG12*, *SAG29*, and *ORE1* were less expressed in an age-dependent manner, as compared to WT plants (**Figures 2A–C**). In addition, the transcript levels of photosynthesis-related genes, such as *RCBS1A* and *CAB1,* were significantly higher in *hos15-2* in an age-dependent manner than in WT plants (**Figures 2D,E**). Taken together, these results suggest that HOS15 regulates senescence in an age-dependent manner through the modulation of senescence- and photosynthesis-related genes.

Loss-of-Function HOS15 Mutant Delays Leaf Yellowing During Dark-Induced Senescence

All stresses have significant effects on leaf senescence but light privation, either as strong darkening or shading, promotes senescence, particularly when a specific plant part is affected (Weaver and Amasino, 2001; Keech et al., 2010). Several PHYTOCHROME INTERACTING FACTORS (PIFs), a basic helix–loop transcription factor family, including PIF3, PIF4, and PIF5, have been reported to promote natural and dark-induced leaf senescence in Arabidopsis (Trivellini et al., 2012; Sakuraba et al., 2014; Song et al., 2014). In the last decade, dark-induced senescence has been widely used for synchronous promotion and other senescence symptoms, such as chlorophyll degradation (Buchanan-Wollaston et al., 2005). As the PWR-HDA9 complex promotes age-triggered and dark-induced senescence (Chen et al., 2016), we therefore assumed that HOS15 protein may also play a role in dark-induced senescence. We examined the dark-induced senescence phenotype of WT, *hos15-2*, CL-1, CL-2 (CL complementation lines expressing *HOS15::HOS15/hos15-2*), and *pwr* and *hda9,* which were used as positive controls. After 4 days of dark treatment, the *hos15-2, pwr,* and *hda9* plants were greener than WT (**Figure 3A**). Next, we exposed the detached leaves to dark stress to observe dark-induced senescence, which was also consistent with the plants' phenotype. The *hos15-2* plant detached leaves were greener than the WT, after 4 days of

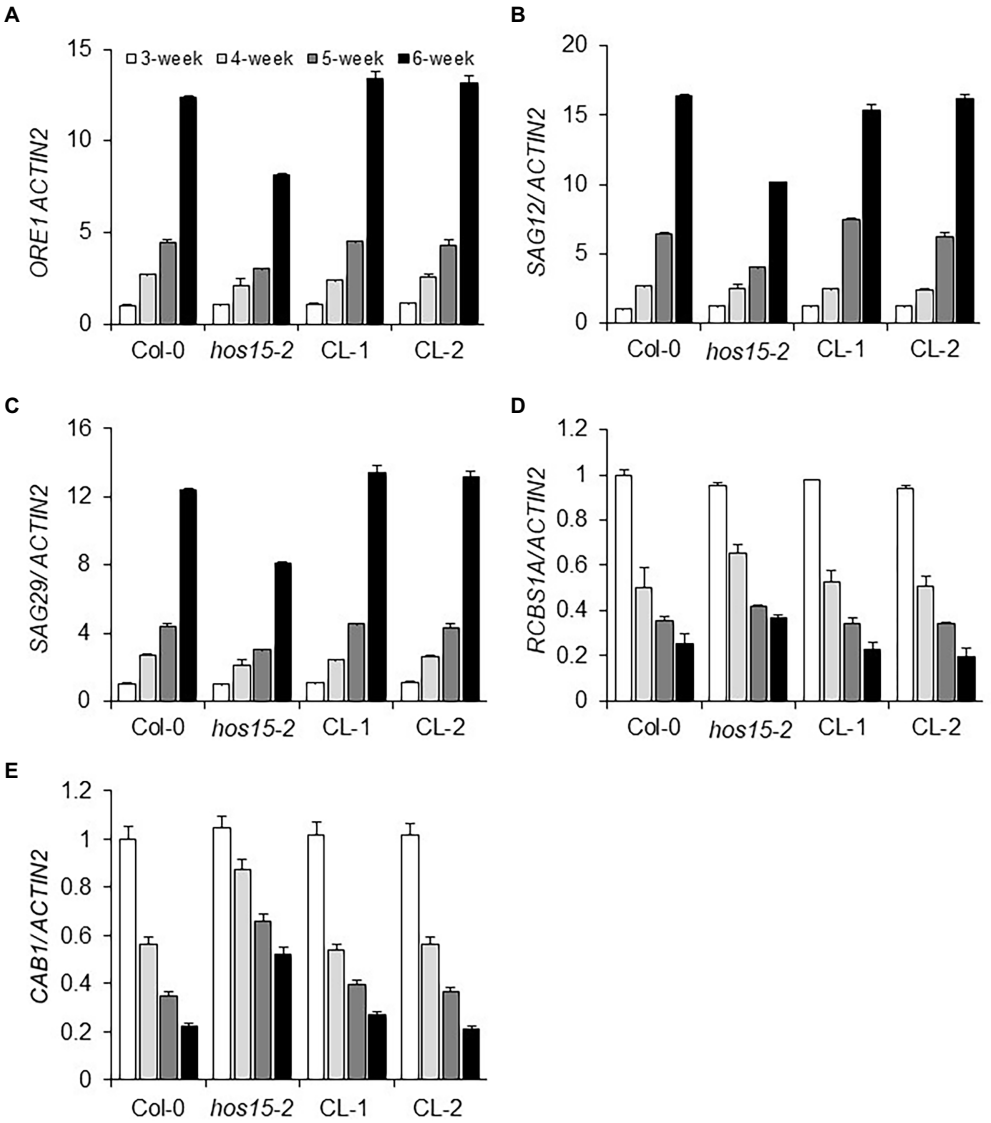

FIGURE 2 | HOS15 differentially regulates senescence- and photosynthesis-related genes in an age-dependent manner. Comparative transcript level analysis of senescence- and photosynthesis-related genes in Col-0 (WT), *hos15-2*, CL-1, and CL-2 plants in an age-dependent manner. **(A–E)** The 3rd and 4th rosette leaves of Col-0, *hos15-2*, CL-1, and CL-2 were sampled at the 3rd, 4th, 5th, and 6th weeks of germination, total RNA was extracted, and cDNA was synthesized. The transcript level of three senescence-related marker genes, *ORE1, SAG12*, and *SAG29*, and two photosynthesis-related genes, *CAB1* and *RBCS1A*, were quantified through qRT-PCR and normalized to 3rd week Col-0. *ACTIN2* was used as the normalization control. Error Bars indicate SD of three independent biological repeats of each sample.

dark stress (**Figure 3B**), suggesting that HOS15 plays a positive role in promoting dark-induced senescence in Arabidopsis. Next, we analyzed the total chlorophyll content in leaves under dark stress, as dark stress was shown to impair chlorophyll content (Chen et al., 2016). The similar leaf senescence phenotypes of *hos15-2*, *pwr,* and *hda9* mutants under dark stress were further supported by their similar retention of chlorophyll content (**Figure 3C**). These observations indicate that HOS15, HDA9, and PWR act in the same pathway to promote leaf senescence.

HOS15 Differentially Regulates the Expression Pattern of Senescence- and Photosynthesis-Related Genes in Dark Stress

Dark-induced senescence remarkably accelerates SAG and NAC-type TF gene expression (Chrost et al., 2004). Darkness also reduces photosynthesis efficiency and the expression levels of photosynthesis-related genes (Eckstein et al., 2019). To assess whether HOS15 regulates the transcript abundance of senescence- and photosynthetic-related genes under dark

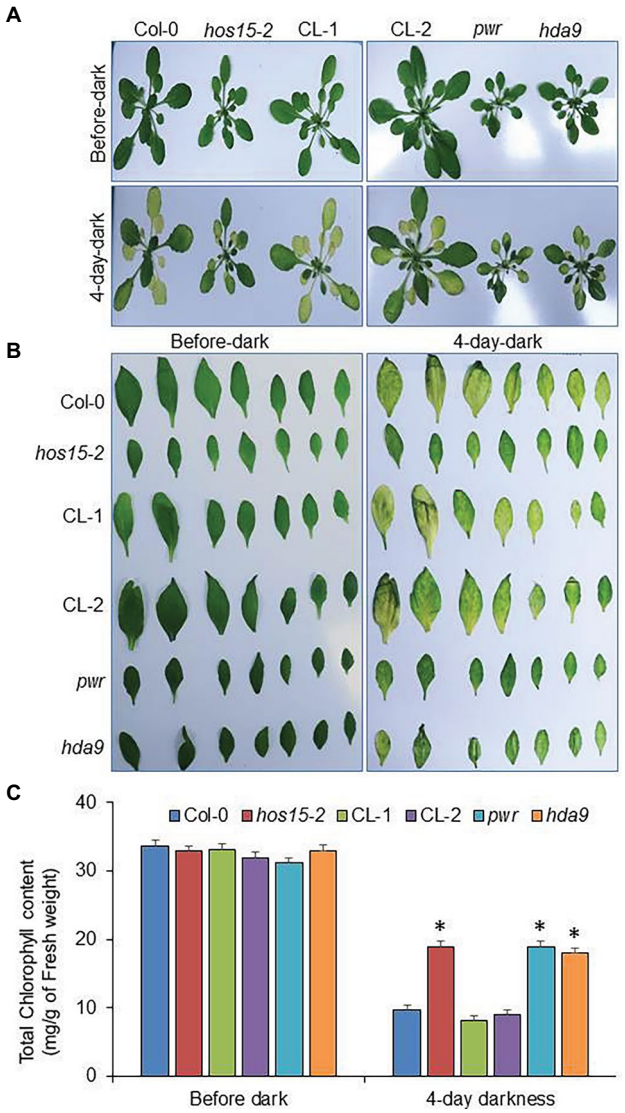

FIGURE 3 | *hos15-2* mutant delays yellowing during dark stress. **(A)** The phenotypic analysis of Col-0 (WT), *hos15-2*, CL-1, CL-2, *pwr*, and *hda9* plants during dark stress. The 4-week-old plants were treated with dark stress for 4 days. **(B)** Dark-induced phenotype of cauline leaves of Col-0, *hos15-2,* CL-1, CL-2, *pwr*, and *hda9* during dark stress. The cauline leaves (1st and 2nd leaves) of the above-mentioned genotypes were introduced to dark stress for 4 days at room temperature conditions. The photographs were taken before and after dark stress. Each experiment was repeated three times with similar results. **(C)** Leaves of the 4-week-old plants of the indicated genotypes (3rd and 4th rosette) were used for measurement of chlorophyll under control condition and in the presence of 4-day dark stress. Error bars show SD. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$).

stress, we tested the transcript abundance of senescence-related genes, such as *SAG12, SAG29*, and *ORE1*, and photosynthesis-related genes, such as *CAB1* and *RCBS1A*. Under dark stress, the transcript levels of *SAG12, SAG29*, and *ORE1* were dramatically reduced in *hos15-2* compared to WT plants (**Figures 4A–C**). In contrast, *CAB1* and *RCBS1A* were dramatically upregulated in *hos15-2* plants compared to WT under dark stress (**Figures 4D,E**). These findings suggest that HOS15 promotes dark-induced senescence through the differential regulation of senescence- and photosynthesis-related genes.

HOS15 Together With PWR-HDA9 Complex Co-regulates Senescence

Recently, the PWR-HDA9 complex has been shown to regulate leaf senescence, as their mutations cause age-triggered and dark-induced senescence phenotypes (Chen et al., 2016). HOS15 has also been reported to interact with and work in the same complex with HDA9-PWR to regulate plant growth and development (Park et al., 2018b; Mayer et al., 2019). To test the genetic relationship between HOS15 and the HDA9-PWR complex in the regulation of plant leaf senescence, we generated double and triple mutants (*hos15pwr*, *hos15hda9*, and *hos15pwrhda9*). As expected, the loss-of-function HOS15, PWR, and HDA9 individually, and their double and triple mutants (*hos15pwr*, *hos15hda9*, and *hos15pwrhda9*) showed late senescence phenotypes compared to WT plants (**Figure 5A**). It has been shown that PWR recruits HDA9 to W-Box-containing genes, *APG9* (autophagy), *WRKY57* (jasmonic acid), and *NPX1* (ABA catabolism), which negatively regulate their transcription and promote senescence (Chen et al., 2016). To investigate whether HOS15 also regulates the same group of genes during senescence, we evaluated the expression levels of *NPX1, APG9*, and *WRKY57*. Compared to WT, transcript levels of *NPX1, APG9*, and *WRKY57* genes were significantly upregulated in *hos15-2*, *hos15pwr*, *hos15hda9*, and *hos15pwrhda9* mutants (**Figures 5B–D**). Late senescence phenotypes of *hos15-2*, *pwr*, *hda9*, *hos15/pwr*, *hos15/hda9* and *hos15/pwr/hda9* mutants were further supported by their similar retention of chlorophyll content (**Figure 5E**). These results demonstrated that HOS15, PWR, and HAD9 might work together in the same complex to regulate leaf senescence by regulating the same group of genes.

Regulation of H3 Acetylation Status Is Essential for Age-Dependent Leaf Senescence

Previous reports have shown that PWR-HDA9 modulates H3 acetylation status and that their mutation results in increased H3 acetylation levels (Chen et al., 2016; Khan et al., 2020; Lim et al., 2020). Similarly, HOS15 is also involved in the regulation of H3 acetylation status through different signaling pathways (Park et al., 2018a; Mayer et al., 2019). To investigate the combined role of this co-repressor complex in histone modulation, we assessed H3 acetylation status in *hos15, hda9,* and *pwr* (single, double, and triple) mutants. As expected, the acetylation levels of H3 (AcH3), H3K9 (AcH3K9), and acetylated lysine (AcK) were remarkably induced in *hos15-2, pwr, hda9, hos15/pwr, hos15/hda9*, and *hos15/pwr/hda9* mutants as compared to WT plants, suggesting

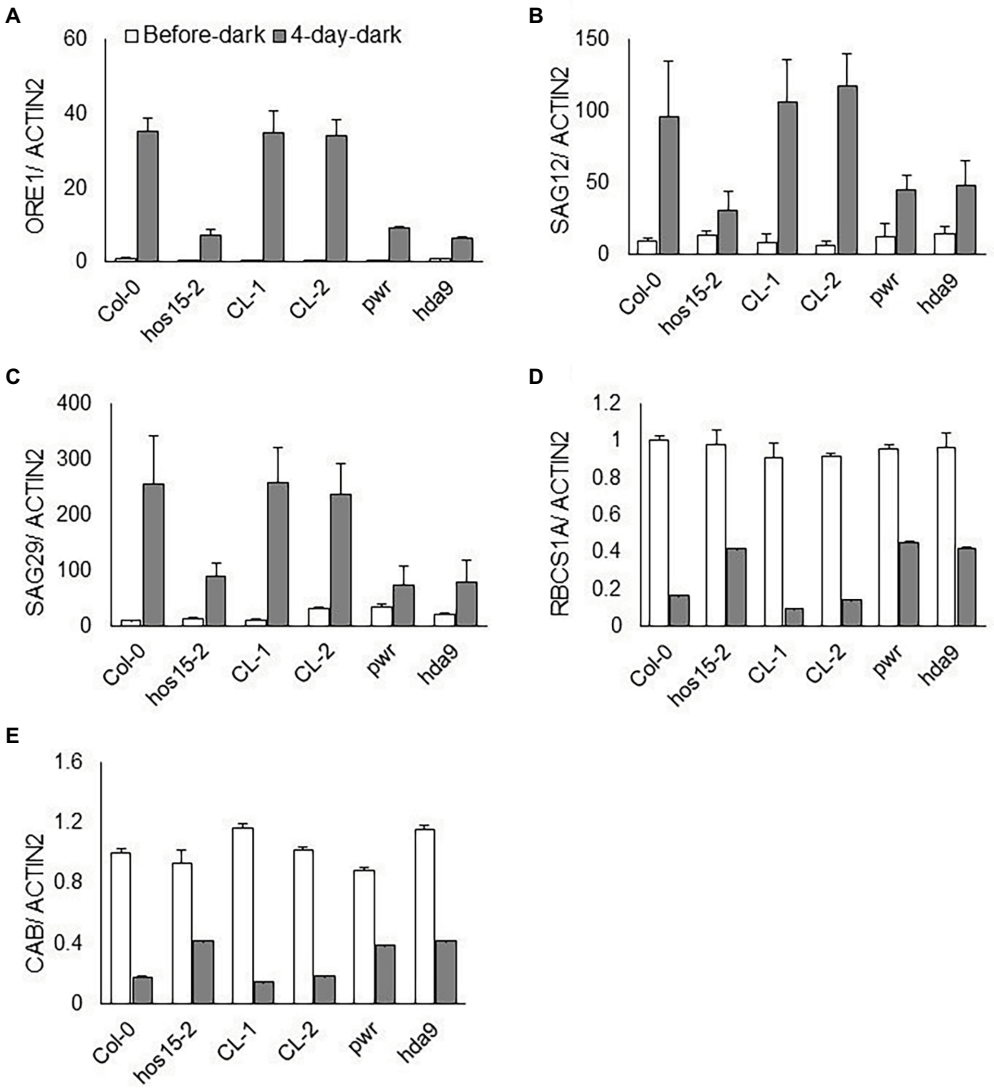

FIGURE 4 | HOS15 differentially regulates senescence- and photosynthesis-related genes during dark-induced senescence. Comparative expression level analysis of senescence- and photosynthesis-related genes. **(A–E)** The transcript level of senescence-related genes *ORE1*, *SAG12*, and *SAG29* and of photosynthesis-related *CAB1* and *RBCS1A* genes before and after dark stress. Cauline leaves of 24-day-old plants were sampled before and after 4 days of dark stress for RNA extraction. The transcript levels were quantified through qRT-PCR and normalized to Col-0 before dark stress. *ACTIN2* was used as the normalization control. Error bars show SD of independent means of three biological repeats.

that the HOS15-PWR-HDA9 complex represses H3 acetylation (**Supplementary Figure 3A**).

Leaf aging and environmental stresses have been considered to play a dynamic role in plant senescence regulation. However, how aging and environmental stresses, particularly dark stress, regulate histone acetylation status remains elusive. Recently, JMJ16, an Arabidopsis JmjC domain-containing protein and H3K4-specific demethylase, was found to repress age-dependent plant leaf senescence through its demethylase function (Liu et al., 2019). To elucidate the effect of leaf aging on histone acetylation status, we determined the H3 acetylation status in an age-dependent manner (YL = young leaves, ML = mature leaves, ES = early senescence, and LS = late senescence; Previously shown by). We found that age triggered a dramatic reduction in H3 acetylation status, particularly H3K9. For instance, acetylation levels in YL and ML were abundantly accumulated as compared to LS leaves (**Figures 6A,B**), suggesting that plant aging (senescence) reduced H3 acetylation status to promote leaf senescence.

HOS15 Negatively Regulates H3 Acetylation Status to Promote Dark-Induced Senescence

Recently, high light (HL) was found to increase the acetylation status of H3 (Guo et al., 2008). As *hos15-2* shows delayed

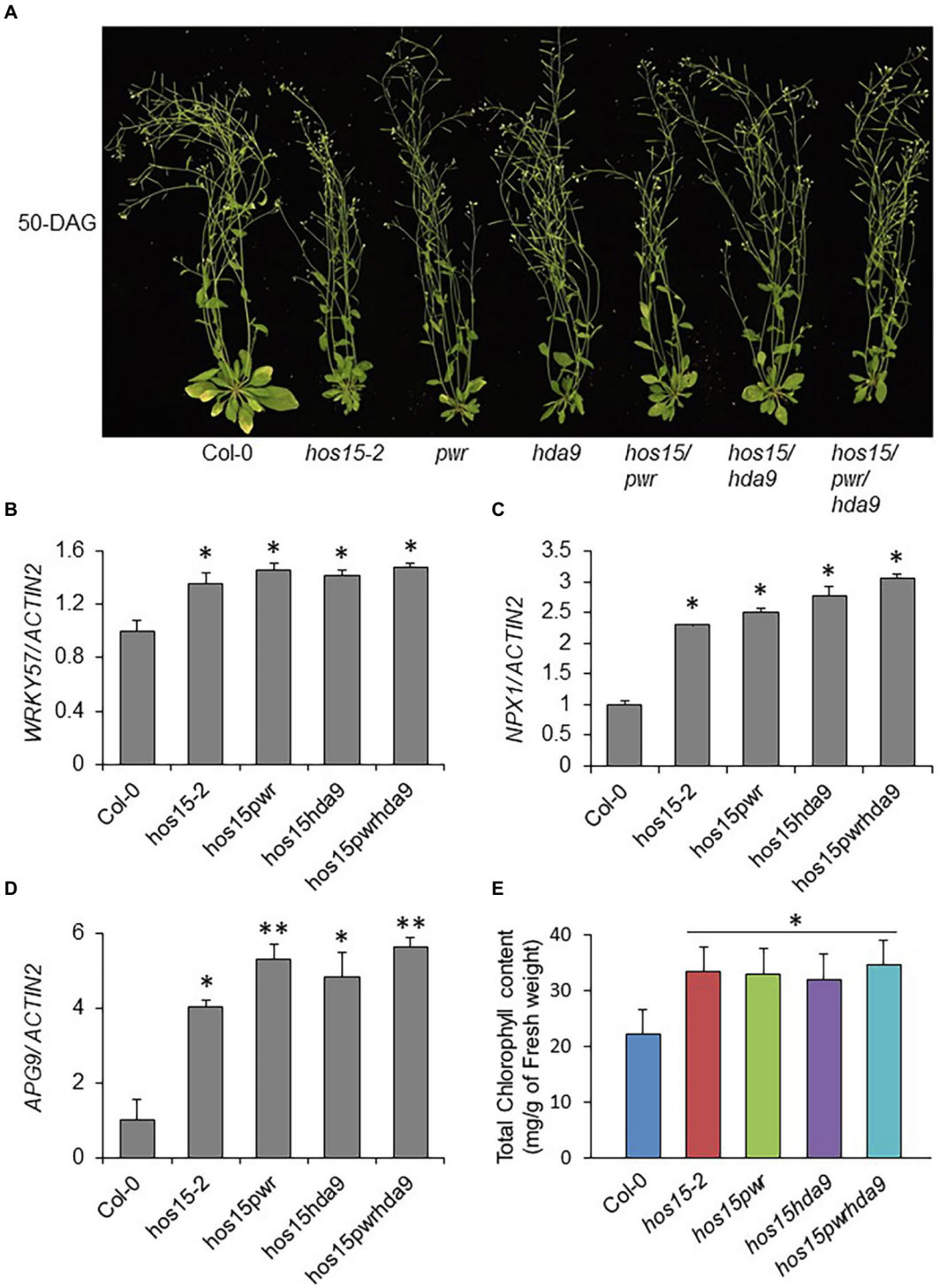

FIGURE 5 | HOS15-PWR-HDA9 complex promotes senescence through the regulation of same group genes. **(A)** Loss-of-function of HOS15-PWR-HDA9 complex components and its double and triple mutants showing late senescence phenotype after 50 days of germination. Seeds of Col-0, *hos15-2, pwr, hda9, hos15pwr, hos15hda9*, and *hos15pwrhda9* plants were grown on MS-media for 12 days and then transferred to soil. **(B–D)** The transcript analysis of senescence negative regulator genes, *WRKY57, NPX1,* and *APG9* after 4 weeks of germination. Total RNA was extracted from the indicated genotypes and cDNAs were synthesized. The expression level was quantified using qRT-PCR. *ACTIN2* was used as the internal control. Error bars represent SD from three biological replicates. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$, $**p < .01$). **(E)** Leaves of the 4-week-old plants of the indicated genotypes (3rd and 4th rosette) were used for measurement of total chlorophyll content. Error bars show SD. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$).

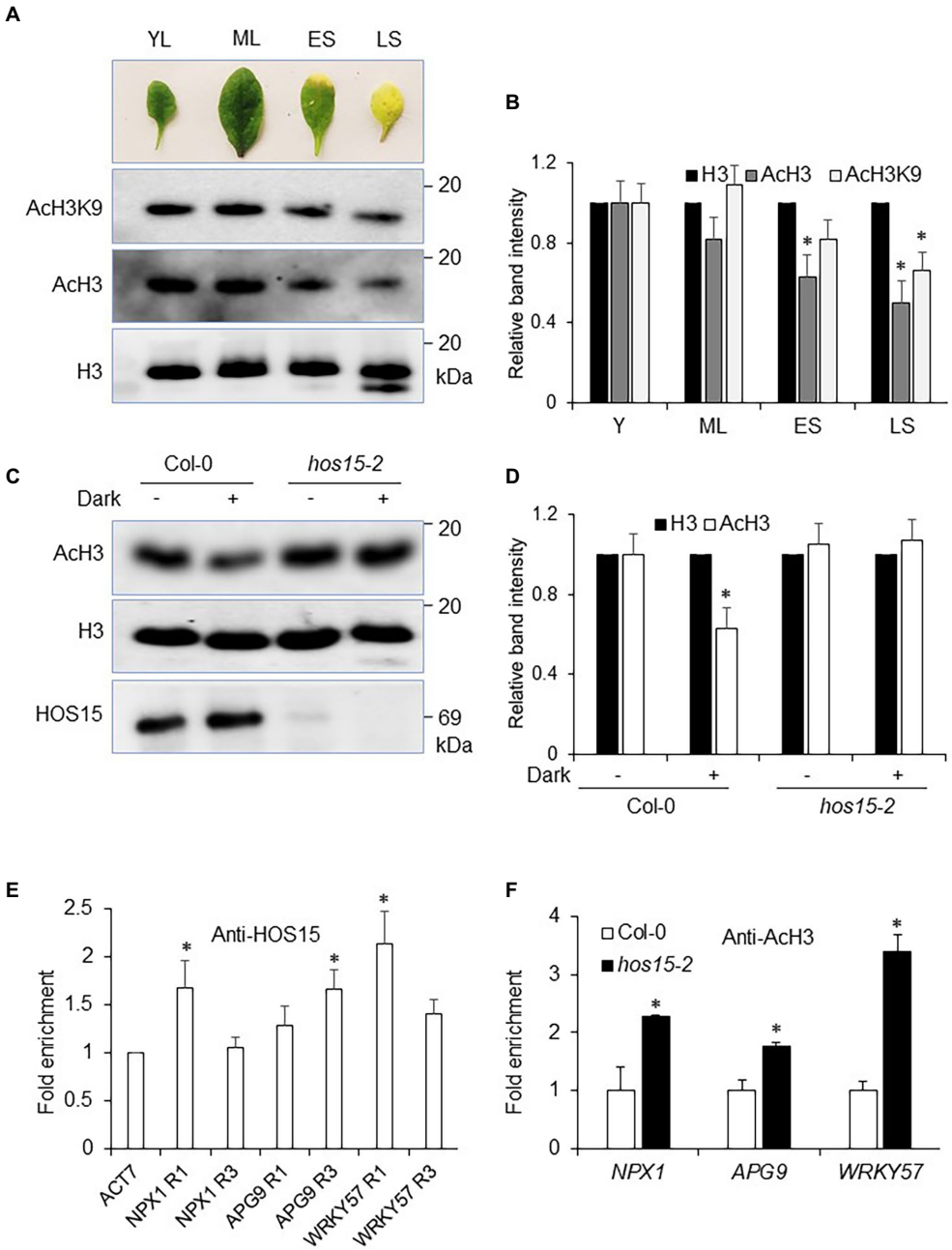

FIGURE 6 | HOS15 represses H3 acetylation status during senescence. **(A)** Plant senescence/aging negatively regulates histone acetylation status. Same age leaves were sampled for nuclear protein extraction. Anti-H3, AcH3, and AcH3K9 antibodies were used to detect the acetylation level in leaves in an age-dependent manner (YL = young leaves, ML = mature leaves, ES = early senescence, and LS = late senescence leaves; previously described by Sun et al., 2017). **(B)** Relative band intensity of H3, AcH3, and AcH3K9 in (A), quantified by ImageJ software, with signal level of H3 set to 1. Error bars represent SE. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$). **(C)** HOS15 regulates dark-induced senescence by affecting H3 acetylation status. Twelve-day-old seedlings of Col-0 and *hos15-2* plants were treated with 4 days of darkness, and then, nuclear proteins were extracted. H3, AcH3 and HOS15 antibodies were used for WB. H3 was used as the loading control. **(D)** Relative band intensity of H3 and AcH3 in **(C)**, quantified by ImageJ software, with signal level of H3 set to 1. Error bars represent SE. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$). **(E)** ChIP assay was performed with anti-HOS15 antibody in Col-0 (WT) plants. *hos15-2* plants were used as negative control. The amount of DNA in the immunoprecipitated complex was determined by RT-qPCR and is presented as the fold enrichment after normalization with control using the ACTIN7 gene promoter. Data represent means (SD) from three biological replicates with three technical repeats. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$). Specific loci used for ChIP assay are described in **Supplementary Figure 4**. **(F)** Chip-qRT-PCR for the leaf senescence negative regulator genes; *NPX1, APG9*, and *WRKY57.* H3 acetylation status were increased in *hos15-2* as compared to Col-0 plants. *ACTIN2* was used as the normalization control. Error bars represent SD of three biological repeats. Significant differences were determined *via* Student's *t*-test (significance: $*p < .05$).

senescence under dark stress, we were interested in exploring the functional role of HOS15 in the modulation of H3 acetylation level during dark stress. As expected, dark stress significantly reduced H3 acetylation in WT (**Figures 6C,D**). In contrast, H3 acetylation levels remained highly stable in *hos15-2* single mutant, *hos15-2/hda9* and *hos15-2/pwr* double mutants, and *hos15-2/hda9/pwr* triple mutant plants regardless of dark stress, suggesting that dark-induced deacetylation requires functional HOS15, HDA9, and PWR (**Figures 6C,D**; **Supplementary Figure 3B**).

According to Chen et al. (2016), PWR and HDA9 directly repress the acetylation status of *NPX1, APG9*, and *WRKY57* to promote leaf senescence. In this regard, we were interested in examining the role of HOS15 in the regulation of *NPX1*, *APG9*, and *WRKY57*, as the expression levels of these genes were found to be dramatically higher in *hos15-2* plants than in WT (**Figure 5B**). We first tested the direct association of HOS15 with the promoters of *NPX1*, *APG9,* and *WRKY57* and found that HOS15 associates with the promoters of these genes at specific regions which were previously identified as target loci for PWR and HDA9 (**Figure 6E**; **Supplementary Figure 4**; Chen et al., 2016). Next, we analyzed H3 acetylation level on these specific regions using Anti-AcH3 antibodies. As shown in **Figure 6F**, the promoters of *NPX1, APG9*, and *WRKY57* were hyper-acetylated in loss-of-function HOS15 mutant as compared with WT plants. Taken together, these findings demonstrate that HOS15 regulates senescence by repressing the acetylation levels of *NPX1, APG9*, and *WRKY57* and that HOS15 works together with PWR-HDA9 in the same complex to regulate aging and dark-induced leaf senescence.

DISCUSSION

HOS15, a WD40 Domain Protein, Regulates Senescence Through Aging

HOS15 acts as a multifunctional protein, and it is actively involved in the regulation of plant growth, development, and stress response. In recent years, we have reported the involvement of HOS15 in cold stress, flowering transition, ABA signaling, drought stress, and pathogen responses (Zhu et al., 2008; Park et al., 2018a; Ali et al., 2019; Mayer et al., 2019; Park et al., 2019; Shen et al., 2020). Interestingly, Mayer et al. (2019) reported that cell division and light-responsive genes were downregulated in the *hda9/hos15* double mutant. Here, we report that HOS15 is also involved in the regulation of senescence through aging and darkness. *hos15-2* plants showed a late senescence phenotype compared to WT after 40 days of germination (**Figure 1**). Aside from exogenous stresses, senescence generally depends on the leaf age and developmental stage (Zentgraf et al., 2004). We also found that *hos15-2* showed a late leaf senescence phenotype compared to WT (**Figure 1B**). Due to senescence, expression levels of some genes were classified into up- and downregulated; SAGs and senescence downregulated genes (SDGs), respectively (Lohman et al., 1994). In this diverse network, we chose two upregulated SAGs, SAG12, and SAG29, as senescence marker genes. Similar to PWR and HDA9, HOS15 also positively regulated the SAG12/29 transcript levels (Chen et al., 2016; **Figure 2**), suggesting that HOS15 plays a positive role in the regulation of these markers during senescence. We found that the expression levels of SAG12 and SAG29 were significantly lower in *hos15-2* than in WT plants after 40 days of germination (**Figure 2**). Moreover, HOS15 also plays a negative role in the regulation of total chlorophyll content in plants, as the total chlorophyll content was found to be much higher in *hos15-2* plants than in WT plants (**Figure 1E**). Taken together, these results indicate that HOS15 acts as a positive regulator of leaf senescence.

Leaf senescence is also involved in chromatin modification, which leads to changes in gene expression patterns. Natural leaf senescence is mainly recognized by aging, which can be triggered by environmental cues and nutritional signals, such as phytohormones, oxidants, abiotic and biotic stresses, and darkness (Mayta et al., 2019). PWR-HDA9-HOS15 work together in the same suppressor complex to regulate plant development and morphological processes (Mayer et al., 2019). Several genes have been identified and reported to be involved in the regulation of plant senescence in an age-dependent manner. Interestingly, PWR and HDA9 promote senescence through aging (Chen et al., 2016), and we expected that HOS15 would be involved in senescence. We found that the expression of HOS15 was gradually induced by age in the 3rd and 4th rosette leaves (**Supplementary Figures 2A,B**). We also evaluated the transcript level of the senescence marker gene *SAG12*, which was less expressed in *hos15-2*, compared to WT, in an age-dependent manner (**Supplementary Figures 2A,B**). We observed that the SAG12 transcript level was low and appeared almost 2 weeks later in *hos15-2*, as SAG12 mRNA upregulation appeared 4 weeks after germination in WT and 6 weeks after germination in *hos15-2* (**Figure 2A**). This evidenced that HOS15 participates as an activator of leaf senescence in an age-dependent manner. In line with this, *hos15-2* showed a late senescence phenotype after 35, 45, and 60 days of germination, respectively, compared to WT (**Supplementary Figure 1**). Like other senescence events in plants, leaf senescence is the final stage of development and decadence from maturation in the history of leaf life (Lim and Hong, 2007). Overall, *hos15-2* rosette leaves of increasing age (older to younger) were greener than that of WT plants (**Supplementary Figure 1**). Hence, based on phenotypes, we showed that HOS15 plays a positive role in leaf senescence regulation through the aging process. In addition, the NAC-type TF family plays an important role in promoting leaf senescence. Kim et al. (2009) reported that *ore1* delayed senescence by aging. In the HOS15 loss-of-function mutant, the ORE1 transcript level was significantly downregulated compared to that in the WT in an age-dependent manner (**Figure 2**). RCBS1A and CAB1 play crucial roles during photosynthesis and CO_2 fixation (Izumi et al., 2012; Bresson et al., 2018). The expression levels of *RCBS1A* and *CAB1* were significantly

elevated in the HOS15 loss-of-function mutant compared to those in WT in an age-dependent manner (**Figure 2**). Taken together, these phenotypical and genetic assessments revealed that HOS15 positively regulates senescence in an age-dependent manner, promotes the expression of SAGs, and suppresses that of photosynthesis-related genes.

HOS15 Acts as Senescence Inducer in Dark Stress

Leaf senescence can also be induced by several exogenous factors, such as dark stress (Lim and Hong, 2007). Interestingly, HAD9 and PWR induce dark-induced leaf senescence (Chen et al., 2016); therefore, we hypothesized that HOS15 might also play a positive role in the regulation of dark-induced leaf senescence. We scrutinized the detached leaf yellowing and found that leaf yellowing was attenuated in *hos15-2* as compared to WT, after 4 days of dark treatment (**Figure 3**), suggesting that HOS15 acts as a positive regulator in dark-induced senescence. Furthermore, in response to 4 days of darkness, the transcript levels of SAG12/29 and ORE1 were significantly less induced in the loss-of-function HOS15 mutant than in the WT (**Figures 4A–C**). In contrast, the expression levels of *RCBS1A* and *CAB1*, photosynthesis-related genes, were higher in *hos15-2* than in WT under dark stress (**Figures 4D,E**). Taken together, these data suggest that HOS15 promotes dark-induced leaf senescence by positively regulating senescence-related genes and negatively regulating photosynthesis-related genes.

HOS15-PWR-HDA9 Complex Regulates the Same Group of Genes Involved in Senescence

HOS15, PWR, and HDA9 have been reported to be predominantly localized in the nucleus. Particularly, PWR has been shown to be involved in the promotion of several micro-RNA genes, which are involved in the regulation of senescence. In addition, a number of ABA signaling (Gao et al., 2016), oxidative stress (Du et al., 2008), jasmonic acid (Jiang et al., 2014), salicylic acid (Veronese et al., 2006), and autophagy pathway (Wang et al., 2011b) genes have been reported to mediate the regulation of plant senescence (Chen et al., 2016). It has also been shown that PWR and HDA9 are involved in leaf senescence through regulation of *APG9, NPX1,* and *WRKY57* genes (Chen et al., 2016). We found that *NPX1, APG9,* and *WRKY57* were significantly upregulated in *hos15-2*, *hos15/pwr, had15/hda9*, and *hhad5/pwr/hda9* mutants as compared to WT (**Figure 5A**), suggesting that HOS15 and PWR-HDA9 work in the same complex to promote leaf senescence in Arabidopsis.

HOS15 Promotes Age-Dependent and Dark-Induced Senescence Through Regulation of Chromatin Structure of NPX1, APG9, and WRKY57

Histone acetylation is often associated with active transcription and open chromatin, whereas deacetylation is generally considered an inactive and compact structure to repress transcription (Verdin and Ott, 2015). PWR-HDA9 modulates the H3 acetylation status and its mutation results in increased acetylation levels (Chen et al., 2016; Khan et al., 2020; Lim et al., 2020). Similarly, HOS15 is also involved in the regulation of histone status through different signaling pathways (Mayer et al., 2019; Park et al., 2019). The physical association of HOS15with PWR and HDA9 led us to investigate the role of HOS15 in the regulation of histone status (acetylation/deacetylation; Mayer et al., 2019; Lim et al., 2020). We found that the acetylation levels of H3 (AcH3), H3K9 (AcH3K9), and acetylated lysine (AcK) were remarkably increased in *hos15-2, pwr, hda9,* and their double and triple mutants compared to WT (**Supplementary Figure 3A**). These observations suggest that the HOS15-PWR-HDA9 complex represses histone acetylation. Leaf aging and dark stress play a vital role in the regulation of leaf senescence. To elucidate the effect of leaf aging on histone acetylation status, we assessed the acetylation level of H3, which was dramatically reduced in late senescence as compared to young leaves (**Figure 6A**). These findings suggest that aging decreases H3 acetylation levels and encourages senescence. In contrast, HL increases the H3 acetylation level (Guo et al., 2008). In this study, we observed that unlike HL, dark stress decreases H3 acetylation level in WT, which is largely controlled by HOS15, HDA9, and PWR (**Figures 6C,D**; **Supplementary Figure 3B**), suggesting that HOS15 together with HDA9 and PWR negatively regulates H3 acetylation levels under dark stress to enhance senescence. HOS15 was further found to repress the acetylation status of *NPX1, APG9*, and *WRKY57* promoters to promote leaf senescence. These results strongly support the notion that like HDA9 and PWR, HOS15 also regulates senescence through repression of the same group of genes. Taken together, our results suggest that HOS15 together with the PWR-HDA9 complex regulates aging and dark-induced leaf senescence by modulating histone acetylation status on the promoters of *NPX1, APG9,* and *WRKY57*. Even though the total H3 acetylation level decreases upon senescence (**Figure 6A**) as well as under dark stress (**Figure 6C**; **Supplementary Figure 3B**), H3 acetylation level at the chromatin of specific genes, such as SAG genes, might increases during senescence. According to previous reports, total H3 acetylation level increases in *hos15-2*, *pwr,* and *hda9* mutants (Chen et al., 2016; Mayer et al., 2019). However, recently Lim et al. (2020) reported that even though the total H3 acetylation level increases in *hos15-2* and *pwr* mutants (Mayer et al., 2019), acetylation level at the promoters of specific genes decreases in these mutants. For instance, compared to WT, H3 acetylation level at the promoter of *COR15A* decreases in *hos15* and *pwr* mutants (Lim et al., 2020). These observations suggest that change in total H3 acetylation level is different from H3 acetylation level at certain chromatins. Decrease in H3 acetylation level upon senescence and dark stress represent major goals for future studies.

PROPOSED MODEL

Plant developmental aging and dark stress are the two main factors that coordinately regulate leaf senescence. In Arabidopsis, HOS15-PWR-HDA9 work in the same corepressor complex

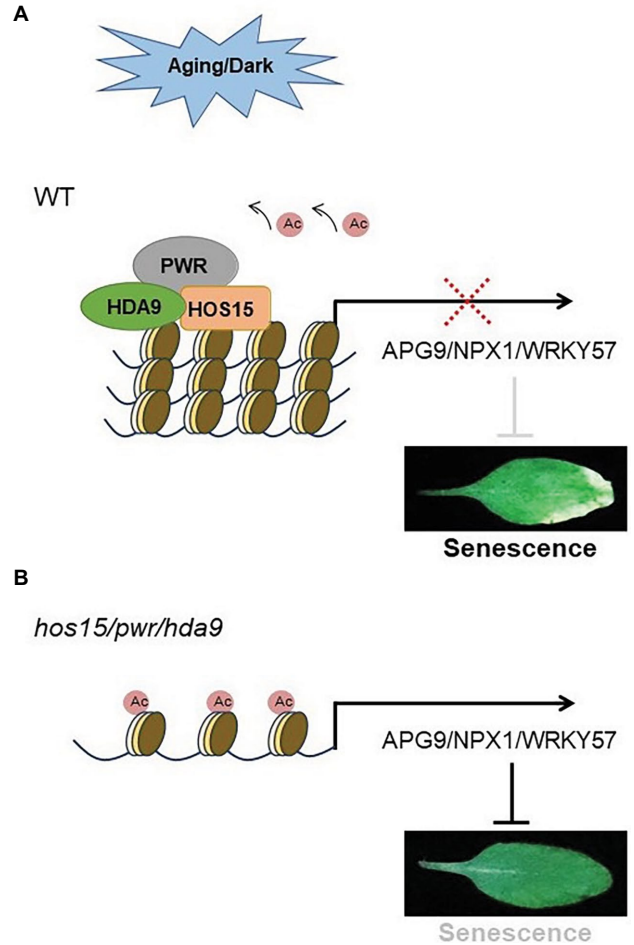

FIGURE 7 | Proposed model. **(A)** *HOS15* works with HDA9/PWR in the same complex, which positively regulates plant senescence through developmental aging process and dark stress. In the developmental aging process or dark stress, the HOS15-PWR-HDA9 complex promotes senescence through the repression of the same target genes, such as *NPX1, APG9*, and *WRKY57*, which are the key negative regulators of plant senescence. **(B)** *NPX1, APG9*, and *WRKY57* are hyperacetylated in plants that lack the HOS15/PWR/HDA9 complex as a whole or any component of this complex. Hyperacetylation of *NPX1, APG9,* and *WRKY57* leads to strong inhibition of age-dependent or dark-induced senescence.

to repress a wide range of genes (**Figure 7**). In our proposed model, HOS15 also acts as a senescence inducer through developmental aging and dark stress, working together with PWR and HDA9 (**Figure 7**). During the developmental aging process and dark stress, HOS15 negatively regulates the acetylation status of the same group of genes, such as *APG9, NPX1*, and *WRKY57* (senescence negative regulators) to modulate plant senescence in Arabidopsis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SZ, AA, and D-JY conceived and designed the experiments, analyzed the data, and wrote the paper. SZ, AA, HK, JP, CL, and Z-YX performed the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.828264/full#supplementary-material

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Rapid Investigation of Functional Roles of Genes in Regulation of Leaf Senescence Using Arabidopsis Protoplasts

*Phan Phuong Thao Doan[1], Jin Hee Kim[2] and Jeongsik Kim[1,2,3]**

[1]Interdisciplinary Graduate Program in Advanced Convergence Technology & Science, Jeju National University, Jeju, South Korea, [2]Subtropical Horticulture Research Institute, Jeju National University, Jeju, South Korea, [3]Faculty of Science Education, Jeju National University, Jeju, South Korea

Leaf senescence is the final stage of leaf development preceding death, which involves a significant cellular metabolic transition from anabolism to catabolism. Several processes during leaf senescence require coordinated regulation by senescence regulatory genes. In this study, we developed a rapid and systematic cellular approach to dissect the functional roles of genes in senescence regulation through their transient expression in Arabidopsis protoplasts. We established and validated this system by monitoring the differential expression of a luciferase-based reporter that was driven by promoters of *SEN4* and *SAG12*, early and late senescence-responsive genes, depending on effectors of known positive and negative senescence regulators. Overexpression of positive senescence regulators, including *ORE1*, *RPK1*, and *RAV1,* increased the expression of both *SEN4*- and *SAG12-LUC* while *ORE7*, a negative senescence regulator decreased their expression. Consistently with overexpression, knockdown of target genes using amiRNAs resulted in opposite *SAG12-LUC* expression patterns. The timing and patterns of reporter responses induced by senescence regulators provided molecular evidence for their distinct kinetic involvement in leaf senescence regulation. Remarkably, *ORE1* and *RPK1* are involved in cell death responses, with more prominent and earlier involvement of *ORE1* than *RPK1*. Consistent with the results in protoplasts, further time series of reactive oxygen species (ROS) and cell death assays using different tobacco transient systems reveal that *ORE1* causes acute cell death and *RPK1* mediates superoxide-dependent intermediate cell death signaling during leaf senescence. Overall, our results indicated that the luciferase-based reporter system in protoplasts is a reliable experimental system that can be effectively used to examine the regulatory roles of Arabidopsis senescence-associated genes.

Keywords: leaf senescence, protoplasts, transient expression, luciferase, cell death, ROS, *Arabidopsis thaliana*

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Sookmyung Women's University, South Korea

***Correspondence:**
Jeongsik Kim
yorus@jejunu.ac.kr

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INTRODUCTION

Leaf senescence is the final and degenerative stage of a leaf's life history; however, it is necessary for plant succession as a beneficial developmental process. Plants coordinate energy and nutrient use among individual leaves when they remobilize resources to newly developing organs or offspring by inducing leaf senescence in old leaves or those having a low photosynthetic

efficiency. Plants determine the fate of leaves to be senesced by reducing photosynthetic activity, dismantling chloroplasts, and changing color, often from green to yellow and/or red. During leaf senescence, the redistribution of nutrients and energy is initially derived from the gradual breakdown of chloroplasts, followed by the catabolism of macromolecules, such as nucleic acids, proteins, and lipids, and degeneration of mitochondria and nuclei (Lim et al., 2007a). For their efficient remobilization, various types of transporters for the molecules composed of carbon, nitrogen, and phosphate are activated (Stigter and Plaxton, 2015; Have et al., 2017). Furthermore, plant leaves sustain self-maintenance activities, such as pathogen defense and detoxification of reactive oxygen species to complete their redistribution activities (Lim et al., 2003; Koyama, 2018).

Plants evolve sophisticated genetic programs for determining the appropriate senescence onset and coordinating senescence progression. The onset of senescence is triggered by various endogenous and environmental signals through the coordinated actions of multiple senescence induction pathways (Woo et al., 2019; Camargo Rodriguez, 2021). A well-studied genetic pathway for senescence onset in Arabidopsis is the trifurcate death circuit consisting of ORE1, EIN2 as an ORE1 activator, and miRNA164 as an ORE1 repressor. ORE1 is a crucial genetic factor to determine leaf senescence onset, and its activation in an aged tissue is inevitable due to EIN2-mediated direct activation or release of miRNA164 repression (Kim et al., 2009). Another example is the protein trio RPK1-CaM4-RbohF, which regulates the transient superoxide production to trigger age- and ABA-dependent leaf senescence and cell death (Lee et al., 2011; Koo et al., 2017).

The onset of leaf senescence by senescence regulatory genes induces changes in the expression of diverse executive senescence-associated genes (SAGs) for the systemic progression of biochemical and physiological processes during leaf senescence. For example, *SEN4* and *SAG12* encode xyloglucan endotransglucosylase/hydrolase 24 and cysteine protease, respectively, which are mainly involved in macromolecule degradation and are upregulated during senescence (Woo et al., 2001; Lim et al., 2003). Conversely, the expression of chlorophyll a/b binding protein gene and rubisco small subunit gene encoding the subunit of light-harvesting complex and rubisco, respectively, declines with senescence progression (Woo et al., 2001). The senescence-associated expression of SAGs is coordinated by a time-dependent involvement of multiple positive and negative senescence regulatory elements, including transcription factors, ncRNA, and signaling components (Woo et al., 2016). Members of NAC and WRKY, two of major senescence-associated transcription factor families are expressed with various temporal patterns along with aging and play positive or negative roles in senescence regulation by regulating the timely expression of SAGs, including genes in the same gene family (Hickman et al., 2013; Kim et al., 2014; Woo et al., 2016; Li et al., 2018). Additionally, a comprehensive study investigating the temporal involvement of senescence regulators identified dynamic changes in NAC regulatory hubs along with leaf aging (Kim et al., 2018a). Time-course expression of NACs in 49 NAC mutants revealed the transition of NAC hubs and their regulatory modules from mature to middle senescent stages and NAC troika was highlighted as a critical hub at the presenescent stage that predominantly repressed the expression of SAGs involved in SA- and reactive oxygen species (ROS) dependent responses.

The temporal and kinetic response analyses aid in understanding gene properties and functions, which further help to elucidate the functional role of genetic pathways (Kim et al., 2018b; Woo et al., 2019). Transient gene expression assays using leaf mesophyll protoplasts are widely used as one of the most efficient approaches for characterizing the cellular functions and regulatory networks of genes in plants in a relatively short time (Rolland, 2018; Domozych et al., 2020). It has contributed to the dissection of signaling pathways in responses to plant hormones or environmental factors (Hwang and Sheen, 2001; Yoo et al., 2007; Li et al., 2019; Lehmann et al., 2020). Recently, knockdown approaches using either RNAi or artificial microRNA (amiRNA) have enabled a reduction in the expression of the endogenous target gene in protoplasts, extending the application of this technology to evaluate gene knockdown effects (Ossowski et al., 2008; Kim and Somers, 2010; Zhang et al., 2019; Vachova et al., 2020). Furthermore, the protoplast viability extension has enabled the investigation of long-term kinetic molecular responses using a luciferase-based reporter for circadian biology (Kim and Somers, 2010). Although leaf senescence is recognized as a long-term developmental event that is controlled by a complex temporal interaction of regulatory components, kinetic analyses using protoplasts have not been applied to plant senescence studies.

In this study, we established a rapid and efficient approach to rapidly dissect the functional roles of genes in leaf senescence regulation through their transient expression using Arabidopsis mesophyll protoplasts. We used overexpression and knockdown approaches to guide the expression of target genes and discovered an altered expression of target genes and SAG reporters, demonstrating its feasibility for investigating a potential regulatory role of senescence regulatory genes at the cellular level. Moreover, these approaches, coupled with histochemical analysis, can reveal distinct and convergent *ORE1* and *RPK1* functions in mediating ROS responses during leaf senescence.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0 wild-type and tobacco (*Nicotiana benthamiana*) plants were sown in pots and grown in an environmentally controlled culture room under LD conditions at 22°C (16 h light/8 h dark cycle; cool white fluorescent bulb with 100–150 μmol m^2 s^{-1}; TLD/840RS; and Philips).

Plasmid Construction

We generated plasmid constructs for transient gene expression in protoplasts or tobacco using GATEWAY cloning technology (Invitrogen, United States). For the reporter plasmids (*SEN4*- and *SAG12-LUC*), the 5′ upstream regions encompassing the *SEN4* (At4g30270) and *SAG12* (At5g45890) promoters of 1.38 kb and 1.53 kb in length, respectively, were amplified by polymerase chain reaction (PCR) with Pfu DNA polymerase, using

Arabidopsis Col-0 genomic DNA as a template and appropriate sets of primers (**Supplementary Table 1**). We subcloned the amplified DNA fragments into the entry vector of pCR-CCD-R using the corresponding restriction enzymes to produce entry clones. The promoter-LUC final constructs were established by LR recombination using the corresponding entry clones and gateway version of the pOmegaLUC_SK+ vector (Kim and Somers, 2010). We used a renilla luciferase (RLUC) under the control of 35S promoter (35S-RLUC) as the transfection control. For the overexpression effectors, the full-length coding sequence of *ORE1* (At5g39610), *ORE7* (At1g20900), *RAV1* (At1g13260), and *RPK1* (At1g69270) was amplified using PCR from Arabidopsis cDNA pools with Pfu DNA polymerase and gene-specific primers (**Supplementary Table 1**). Further, we subcloned the amplified DNA fragments into the pCR-CCD-F entry vector to produce entry clones. Then, we recombined the entry clones using gateway versions of pCsVMV-eGFP-N-999 and pCsVMV-eGFP-N-1300 to produce the effector plasmids of ORE1-, RAV1-, RPK1-, and ORE7-pCsVMV-eGFP-N-999 and binary plasmids of ORE1-, RAV1-, RPK1-, and ORE7-pCsVMV-eGFP-N-1300, respectively. For amiRNA effector, ORE1 amiRNA plasmid was generated by digesting pAmiR-ORE1 (CSHL_075023) with *Pst*I and *Bam*HI, then, ligating each resulting amiRNA foldback fragment into *Pst*I/*Bam*HI digested pCsVMV-PP2C-AmiR vector (Kim and Somers, 2010). We designed RPK1 amiRNA using the Web MicroRNA Designer 3[1] as previously described (Schwab et al., 2006; Kim and Somers, 2010). The amiRNA foldback fragments were generated by overlapping PCR using the pCsVMV-PP2C-AmiR plasmid as a template and the designated primers for each construct (**Supplementary Table 1**). All resulting PCR fragments containing the full amiRNA foldback were cloned downstream of the CsVMV promoter into unique *Pst*I and *BamH*I restriction sites of pCsVMV-AmiR. Also, we utilized the pCsVMV-AmiR plasmid as a transfection control.

Protoplast Isolation and Transfection

We conducted protoplast isolation and DNA transfection as previously described (Kim and Somers, 2010). Briefly, 10 to 15 leaves of three- to four-week-old Col-0 plants were sterilized with 70% ethanol for 30 s, and then rinsed with sterile water twice. Leaves scratched briefly with sandpaper were incubated in 10 ml of enzyme solution (1% Cellulase R10, 0.5% Macerozyme R10 [Yakult Honsha, Japan], 400 mM mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES-KOH [pH 5.7], and 0.1% BSA [Sigma A6793, United States]) for 2.5 h by gentle shaking at room temperature. Protoplasts released into enzyme solutions were filtered and harvested into a round-shaped culture tube by centrifugation at 100 g for 5 min. We resuspended the protoplast pellets in 2 ml of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 1.5 mM MES-KOH [pH 5.7], and 5 mM Glucose), and placed them on ice for 30 min. The protoplasts were harvested and resuspended in MMG solution (400 mM mannitol, 15 mM $MgCl_2$, and 4 mM MES-KOH [pH 5.7]), and the final cell concentration was adjusted to 2×10^5 ml^{-1}. Also, 30.2 µl of plasmid mixtures with 25 µl effector, 5 µl reporter, and 0.2 µl internal control were transfected into 200 µl of protoplasts in MMG solution. We prepared effector plasmids of overexpression or amiRNA and reporter plasmids by CsCl gradient purification using an ultracentrifuge (in Bio-Health Materials Core-Facility, Jeju National University, Korea), and their DNA concentration was adjusted to 2 µg µl^{-1} per 4 kb DNA. We performed transfections by adding 230 µl (1 vol.) of polyethylene glycol (PEG) solution [40% PEG-4000, 200 mM mannitol, and 100 mM $Ca(NO_3)_2$] into protoplasts containing DNAs, and incubating them for 8–15 min at room temperature. We diluted the protoplast-DNA-PEG mixture with 920 µl (2 vol.) of W5 solution. After centrifugation, the pellets were resuspended in 700 µl of W5 solution containing 5% fetal bovine serum (Sigma F4135, United States) and 50 µg ml^{-1} ampicillin. For RT-PCR analysis from protoplasts, we used 800 µl of protoplasts and 120 µl of amiRNA plasmids for a transfection sample.

Luminescence Measurement

We analyzed the expression of the specific senescence reporters by kinetic measurement of luciferase activity in protoplasts. A 300 µl of transfected protoplasts were transferred into each well containing 3 µl of LUC substrate (5 mM luciferin, Goldbio LUCK-250, Netherlands) or 3 µl of RLUC substrate (10 µM Coelenterazine-native, Sigma C2230, United States) of a white and round bottom 96-well microplate. The microplate was covered with a clear plastic cap and incubated at 22°C in the dark on a GloMax 96 microplate luminometer (Promega, United States). For 3 days, we acquired images every 30 min. In each data set, we determined promoter activities by luciferase activity at the indicated time and normalized them to the maximum RLUC level throughout the measurement. The relative LUC expression was calculated by normalizing to the maximum level of LUC/RLUC throughout the measurement in protoplasts transfected with GFP or the control amiRNA effector during each trial.

RNA Extraction and Quantitative Reverse Transcriptase-PCR

We conducted total RNA extraction and cDNA synthesis, as previously described (Kim and Somers, 2010). Protoplasts were incubated for 16 h after transfection under dim white light and flash-frozen in liquid nitrogen for subsequent RNA extraction and qRT-PCR. Then, we extracted total RNA using WelPrep™ Total RNA Isolation Reagent (Welgene, Korea) and used it for cDNA synthesis using the ImProm II™ Reverse Transcriptase System kit (Promega, United States). Further, qPCR was performed on a CFX96 real-time qPCR detection system (Bio-Rad, United States) using appropriate primer sets. We designed primers for candidate genes using Primer 3 software (Untergasser et al., 2012) and are listed (**Supplementary Table 1**). The relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method (Kim et al., 2008). *ACT2* gene was used as the internal reference.

[1]http://wmd3.weigelworld.org

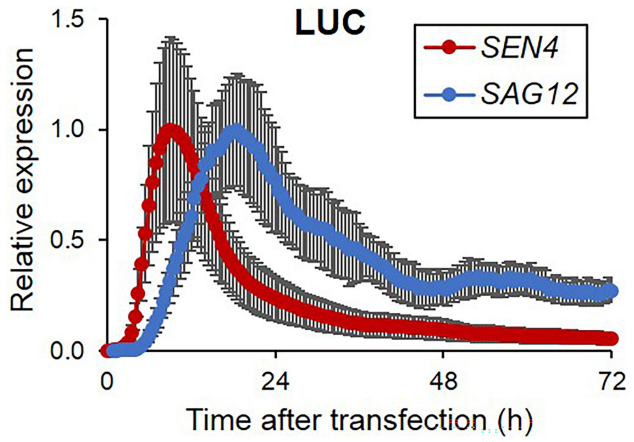

FIGURE 1 | Bioluminescence expression patterns of luciferase reporters controlled by senescence-associated promoters in Arabidopsis protoplasts during darkness. Bioluminescence traces in Arabidopsis protoplasts expressing a firefly luciferase gene (*LUC*) driven by *SEN4* and *SAG12* promoters (*SEN4-* and *SAG12-LUC*) and a Renilla luciferase (RLUC) driven by 35S promoter (35S-RLUC) in darkness after transfection are shown. Image acquisition was performed every 30 min for 3 d. Luciferase activity of LUC was normalized to the maximum level of RLUC over the assay. Each data set was normalized to the maximum level of LUC/RLUC throughout the measurement. Data represent mean ± SE ($n = 3$). Similar results were obtained in two independent trials.

Transient Expression in Tobacco and Histochemical Analysis

We conducted a transient expression in tobacco using P19-enhanced Agrobacteria infiltration (Voinnet et al., 2003). Agrobacteria containing plasmids of ORE1- or RPK1-pCsVMV-eGFP-N-1300 or empty pCsVMV-eGFP-N-1300 were infiltrated into tobacco. Tobacco leaves were incubated at 22°C in the same chamber where plants are cultivated before being harvested on the days indicated after infiltration. Next, we performed a histochemical analysis with minor modifications as described (Yu et al., 2019). For the visualization of H_2O_2 and superoxide accumulation, 3,3′-diaminobenzidine (DAB; Sigma D8001, United States) and nitrotetrazolium blue chloride (NBT; Sigma N6639, United States) were used, respectively. Tobacco leaves transiently expressing *ORE1-GFP*, *RPK1-GFP*, and *GFP* were subjected to DAB and NBT staining at the indicated days. The leaves were soaked in 1 mg ml^{-1} DAB or 2 mg ml^{-1} NBT overnight and boiled for 10 min in 100% ethanol. Stained leaves were stored in 95% ethanol at room temperature before being photographed. Cell death in tobacco leaves was visualized by trypan blue staining. The treated tobacco leaves were completely immersed in 1 mg ml^{-1} trypan blue (Fluka 93590, Switzerland) solution and incubated for at least 30 min at room temperature. The stained leaves were washed immediately with 98–100% ethanol for decolorization and photographed under a bright-field microscope. Intensities of trypan blue, DAB, or NBT staining were quantified using ImageJ software[2] as previously described (Juszczak and Baier, 2014). Stained leaf disks from five leaves expressing *GFP*, *ORE1-GFP*, and *RPK1-GFP* were then used to quantify staining intensity.

[2]https://imagej.nih.gov/ij/

Protoplast Viability Test

Cell death of Arabidopsis protoplasts was assayed by Evans blue staining (Sigma E2129, United States). The protoplasts expressing *GFP*, *ORE1-GFP*, *RAV1-GFP*, *RPK1-GFP*, and *ORE7-GFP* were incubated for 72 h under dim white light. We extracted transfected protoplasts at different time points (0, 6, 24, 48, and 72 h after transfection) and loaded them for 2–3 min with 10 mg ml^{-1} Evans blue dye. We visualized and photographed blue-stained dead cells using Zeiss Axiostar Plus Microscope (Ambastha et al., 2017). Stained protoplasts were counted in five to twelve fields containing at least 50 cells from each sample cell death measurement. Cell death (%) was measured as the following formula: Number of blue cells/Total number of cells × 100%.

RESULTS

Establishment of Luciferase-Based Reporters Controlled by the Promoter of SAGs for Cell-Based Senescence Assay

Arabidopsis protoplasts are an effective experimental system for rapid functional analyses, enabling us to investigate diverse molecular and cellular functions of genes of interest based on responsiveness luciferase reporters through their transient expression (Tyurin et al., 2020). Firstly, to establish the luciferase-based reporters for investigating leaf senescence in protoplasts, we selected two SAGs, including *SAG12* and *SEN4* with increased expression during the dark- and age-induced leaf senescence. Each SAG promoter-driven luciferase reporter construct was transfected individually into Arabidopsis mesophyll protoplasts and we monitored luminescence activity in a time-series manner. As shown in **Figure 1**, the expression of *SEN4-LUC* and *SAG12-LUC* was significantly induced with different accumulation rates and peak periods but was quickly reduced following their peak expression. *SEN4-LUC* expression was induced more rapidly with an earlier peak time than *SAG12-LUC,* which is similar to that observed in intact leaves (**Supplementary Table 2**; Woo et al., 2001, 2002). Additionally, expression levels of *SEN4-* and *SAG12-LUC* were higher than that obtained from the basal expression level of a promoterless-*LUC* reporter (**Supplementary Figure 1**). Peaks of their expression were also different from *35S-LUC*, thereby supporting the potential use of *SEN4-* and *SAG12-LUC* as cellular senescence reporters. We also tested other *LUC* reporters driven by the promoters of *PRK-*, *CA1-*, and *THIONIN-LUC* with downregulated expression during senescence but failed to obtain reliable expression patterns for a reporter assay, although their transcript levels are high in transcriptome analysis during age- and dark-induced senescence (**Supplementary Figure 1**; Woo et al., 2016; Kim et al., 2018c). From these results, we conducted subsequent analyses using only the *SEN4-LUC* and *SAG12-LUC* reporters.

Transient Overexpression of Senescence Regulatory Genes as Effectors

Since the *SEN4*- and *SAG12-LUC* revealed clear and distinct expression patterns in protoplasts, we attempted to evaluate the effect of ectopic overexpression of senescence regulatory genes on the expression of both reporters. We generated overexpression effector constructs for positive (*ORE1*, *RAV1*, and *RPK1*) and negative (*ORE7*) senescence regulators fused to a green-fluorescent protein (GFP) under the control of Cassava vein mosaic virus (CsVMV) promoter. We confirmed that these effector proteins were strongly expressed and exclusively localized in subcellular organelles as reported; ORE1, RAV1, and ORE7 in the nucleus and RPK1 in the plasma membrane (**Supplementary Figure 2**; Lim et al., 2007b; Kim et al., 2009; Woo et al., 2010; Koo et al., 2017). GFP-fused senescence regulator effector plasmids and GFP as a control were co-transfected with *SEN4-LUC* and *SAG12-LUC* reporters, and time-series luminescence levels of the reporters were compared (**Figure 2**). When *GFP* was transiently introduced, the luminescence expression of both reporters was induced with differential expression levels and patterns throughout the assay for 72 h. Ectopic expression of *RPK1* led to a 10- and 18-fold increase in *SEN4-LUC* and *SAG12-LUC* expression at the peak time, respectively, but no significant change in their peak time compared with control *GFP* expression (**Figures 2A,B**; **Supplementary Table 2**). Interestingly, ectopic expression of *ORE1* induced distinct expression patterns of *SEN4*- and *SAG12-LUC* in that their expression was induced earlier and reached maximum levels more rapidly, compared with that of control, and was dampened quickly (**Figures 2C,D,G,H**; **Supplementary Table 2**). However, *RAV1* had a late inducing effect on the expression of *SEN4-LUC* and *SAG12-LUC*, and maintained their expression higher, although both expressions earlier were the same or lower relative to those of the control (**Figures 2E–H**; **Supplementary Table 2**). Overexpression of *ORE7*, as a negative senescence regulator, resulted in a dampened expression of *SAG12-LUC* throughout the assay, but a slight reduction of *SEN4-LUC* expression at a later time of assay ranging from 65 to 72 h (**Figures 2E–H**). These results were consistent with early senescence phenotypes in *ORE1*, *RPK1*, and *RAV1* overexpressors, and delayed senescence phenotypes in *ORE7* overexpressor (Lim et al., 2007b; Kim et al., 2009; Woo et al., 2010; Koo et al., 2017). Hence, our result indicates that the effectiveness of senescence regulators can be evaluated by overexpressing them, and co-expressing *SEN4-LUC* and *SAG12-LUC* reporters through a prolonged protoplast assay.

Transient amiRNA-Mediated Knockdown of the Senescence Regulatory Genes as Effectors

Artificial microRNA (amiRNA)-based knockdown approaches have been widely used for gene function studies in planta or protoplasts as a reverse-genetic approach (Ossowski et al., 2008; Kim and Somers, 2010). As an alternative and complementary approach to transient overexpression, we attempted to explore the feasibility of amiRNA-based knockdown approaches for investigating the functional regulatory role of genes in senescence regulation in protoplasts. We generated amiRNAs targeting *ORE1* and *RPK1* and validated their knockdown effect on endogenous target gene expression by qRT-PCR. *ORE1* and *RPK1* amiRNAs lowered the expression of each corresponding target gene by 41 and 45%, respectively, when compared with the control vector (**Figures 3A,B**). Because *RAV1* and *ORE7* are members of the Arabidopsis large family genes, and plants with loss-of-function or knockdown of *RAV1* and *ORE7* exhibited no senescence phenotypes (Lim et al., 2007b; Woo et al., 2010), the amiRNAs of *RAV1* and *ORE7* were excluded from a pilot test set of amiRNA-based knockdown approach. We examined the effects of *ORE1* and *RPK1* amiRNAs on the expression patterns of both *SEN4*- and *SAG12-LUC* reporters after co-transfection of effectors and reporters in protoplasts. *ORE1* and *RPK1* amiRNAs led to 2.7- and 1.6-fold reduction of *SAG12-LUC* expression at its peak time compared with control, respectively, although no effect on *SEN4-LUC* expression was observed (**Figures 3C,D**; **Supplementary Table 2**). As the reduction effect of *ORE1* and *RPK1* amiRNAs on *SAG12-LUC* is consistent with increased expression of *SAG12-LUC* by *ORE1* and *RPK1* overexpression (**Figure 2**), the amiRNAs approaches in protoplasts can be useful for assessing gene functions in senescence regulation. To further validate amiRNA approaches, we included additional amiRNA effectors of *ORE4* and *ORE9* whose loss-of-function mutants exhibited delayed leaf senescence, along with overexpression of *miR164B* (miR164B-OX), a senescence regulatory miRNA targeting *ORE1* (Woo et al., 2001, 2002; Kim et al., 2009). We failed to detect any significant change in *SEN4-LUC* when *ORE9*, *ORE4* amiRNAs, and miR164B-OX were introduced (**Supplementary Figure 3A**; **Supplementary Table 2**), which are similar when *ORE1* and *RPK1* amiRNAs were used. However, we observed a reduction in *SAG12-LUC* in protoplasts transfected with *ORE9* amiRNA and miR164B-OX, but no change in *SAG12-LUC* levels in protoplasts transfected with *ORE4* amiRNA (**Supplementary Figure 3B**; **Supplementary Table 2**). Since *ORE4* encodes plastid ribosomal small subunit protein 17, and the *ore4-1* mutant had no phenotype in dark-induced senescence, no change in *SAG12-LUC* by *ORE4* amiRNA can be explained. Collectively, these results indicate that the amiRNA-based knockdown approach with *SAG12-LUC* reporter is at least valid for a rapid functional assay of genes involved in senescence regulation, as is the overexpression approach.

Divergent Function of ORE1 and RPK1 in Premature Cell Death Regulation Through Different ROS Signaling

Overexpression of selected senescence regulators led to different kinetic expression patterns of senescence reporters (**Figure 2**), with some of them, such as *ORE1* and *ORE7* inducing dampened expression. Since senescence accompanies death, cell death can induce suppressed or dampened *SEN4*- and *SAG12-LUC*

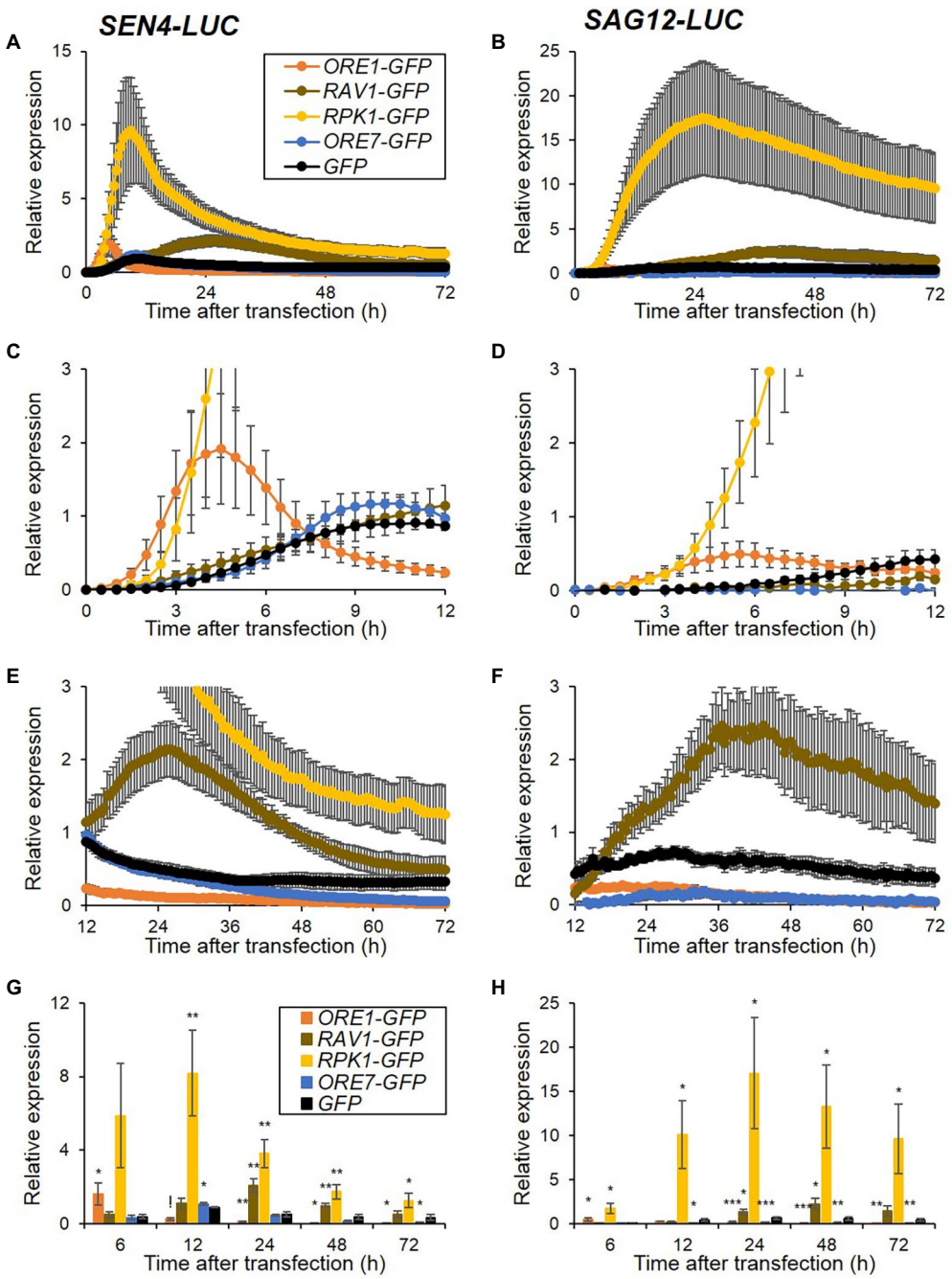

FIGURE 2 | Expression patterns of *SEN4-* **(A)** and *SAG12-LUC* **(B)** reporters by overexpression of senescence regulators. Arabidopsis protoplasts were co-transfected, as described in **Figure 1**, but with overexpression effectors of *GFP*, *ORE1-*, *RAV1-*, *RPK1-*, and *ORE7-GFP* and either reporter of *SEN4-* or *SAG12-LUC*. **(A–F)** Bioluminescence traces of *SEN4-* or *SAG12-LUC* throughout the measurement **(A,B)**, and their traces with an adjusted scale at early (0–12 h; **C,D**) and late (12–72 h; **E,F**) time points are shown. Symbols in **(B–F)** are the same as in **(A)**. Luciferase activity of LUC was normalized to the maximum level of RLUC over the assay. Relative expression of *SEN4-* **(A,C,E)** and *SAG12-LUC* **(B,D,F)** was calculated by normalization to the maximum level of LUC/RLUC throughout the measurement in protoplasts transfected with *GFP* effector in each trial. **(G,H)** Relative expression of *SEN4-* **(G)** and *SAG12-LUC* **(H)** at 6, 12, 24, 48, and 72 h after transfection. Symbols in **(H)** are the same as in **(G)**. Data represent mean ± SE ($n = 6$). A statistical analysis was performed using a two-tailed Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and $^{\dagger}p < 0.0001$).

expression in protoplasts. Therefore, we explored cell death responses in protoplasts where *ORE1*, *RAV1*, *RPK1*, and *ORE7* were overexpressed. We transfected plasmids of overexpression cassettes of *ORE1*, *RAV1*, *RPK1*, and *ORE7* in protoplasts and stained transfected protoplasts with Evans blue dyes in a time-dependent manner. We confirmed that the transfection efficiency

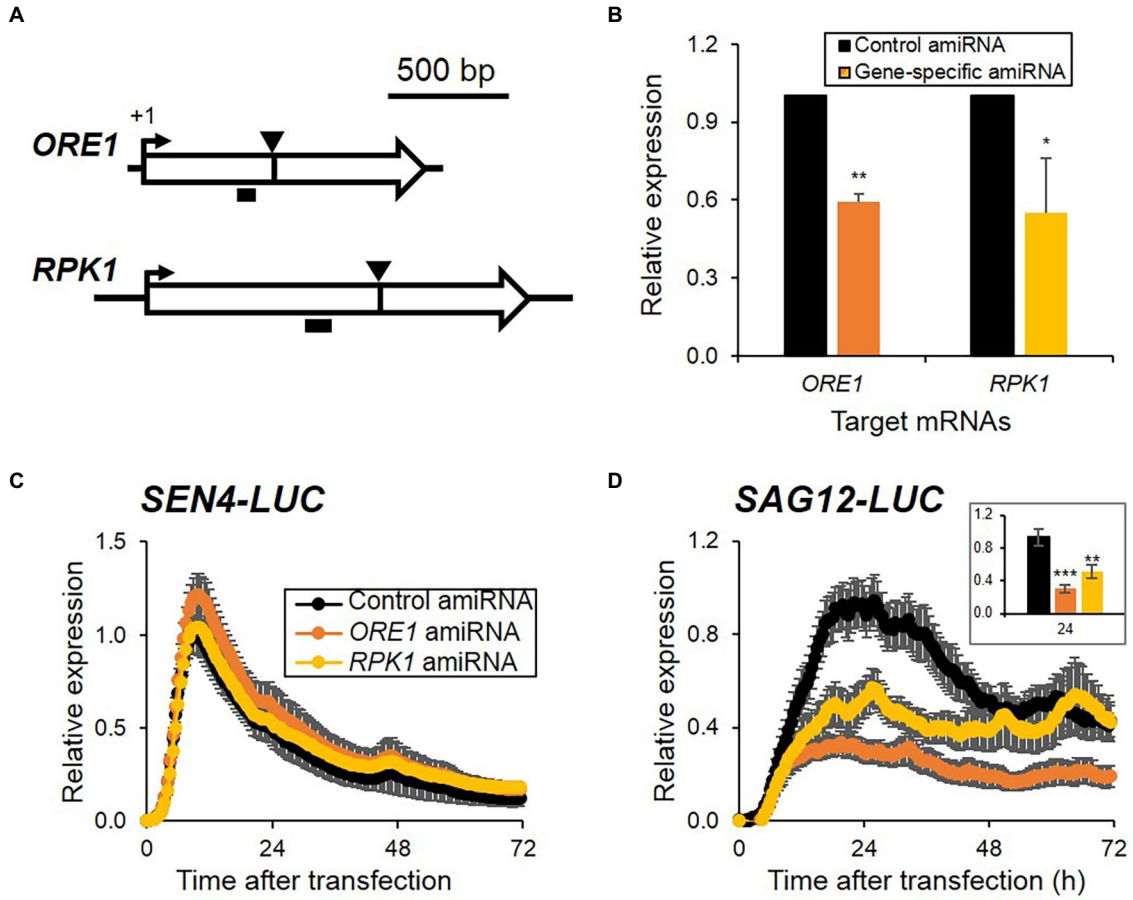

FIGURE 3 | Expression patterns of *SEN4-* **(A)** and *SAG12-LUC* **(B)** reporters by reduced expression of senescence regulators using amiRNAs. **(A)** Schematic of the *ORE1* and *RPK1* with the amiRNA-targeted site (arrowheads) and the PCR amplified region (horizontal black bars) indicated for each. **(B)** Relative expression of *ORE1* and *RPK1* from protoplasts transfected with control or the gene-specific amiRNA indicated and harvested at 16 h incubation under dim light after transfection. Data represent mean ± SE ($n = 3$). **(C,D)** Bioluminescence traces of *SEN4-* **(C)** and *SAG12-LUC* **(D)** with amiRNA-construct gene targeting *ORE1* or *RPK1* during darkness. Symbols in **(D)** are the same as in **(C)**. (Inset) Bar graph of relative expression of *SAG12-LUC* at 24 h after transfection. Luciferase activity of LUC was normalized to the maximum level of RLUC over the assay. Relative expression of *SEN4-* **(C)** and *SAG12-LUC* **(D)** was calculated by normalization to the maximum level of LUC/RLUC throughout the measurement in protoplasts transfected with control amiRNA in each trial. Data represent mean ± SE ($n = 6$). A statistical analysis was performed using a two-tailed Student's *t*-test (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

in our assay was approximately 80% for all effectors, which was higher than 50% of the transfection efficiencies recommended for successful experiments (**Supplementary Figure 4**; Yoo et al., 2007). The extent of cell death accumulation in *ORE1*-overexpressing protoplasts significantly increased to 44.1% at 6 h and remained higher till to 85.0% at 72 h post-transfection, but that of control protoplasts was 19.5% at 6 h and 39.6% at 72 h post-transfection. Interestingly, *RPK1* overexpression at later incubation time points ranging from 48 to 72 h increased the cell death level to 67.0%, which is a similar level to that of *ORE1*. However, the expression of *RAV1* and *ORE7* did not affect cell death accumulation in the transfected protoplasts compared with that of control (**Figure 4**). We also confirmed that all effector proteins were expressed to a certain level up to 72 h, although ORE1 and RPK1 protein levels declined from 24 to 72 h, which was proposed to be due to the increasing proportion of dead cells induced by *ORE1* and *RPK1* overexpression (**Supplementary Figure 5**). These results indicate that the earlier induction of SAG promoters in *ORE1*-overexpressing protoplasts is due to premature cell death of protoplasts by *ORE1*. Furthermore, these results imply that *ORE1* and *RPK1* have different kinetic functions in triggering cell death during senescence.

RPK1 and *ORE1* mediate ROS signaling and/or production to trigger age-dependent cell death (Balazadeh et al., 2010; Koo et al., 2017). Therefore, we dissected the accumulation rate of two major ROS species, H_2O_2, and superoxide, as well as cell death in tobacco tissues that ectopically expressed *RPK1* and *ORE1* (**Figure 5**). Cell death, H_2O_2, and superoxide were visualized using trypan blue, DAB, and NBT, respectively. Trypan blue-mediated cell death assay in tobacco exhibited similar results as shown in protoplasts: *ORE1* overexpression provoked an earlier onset of cell death marked with blue stains in tobacco leaves from 1 day after infiltration (DAI), whereas

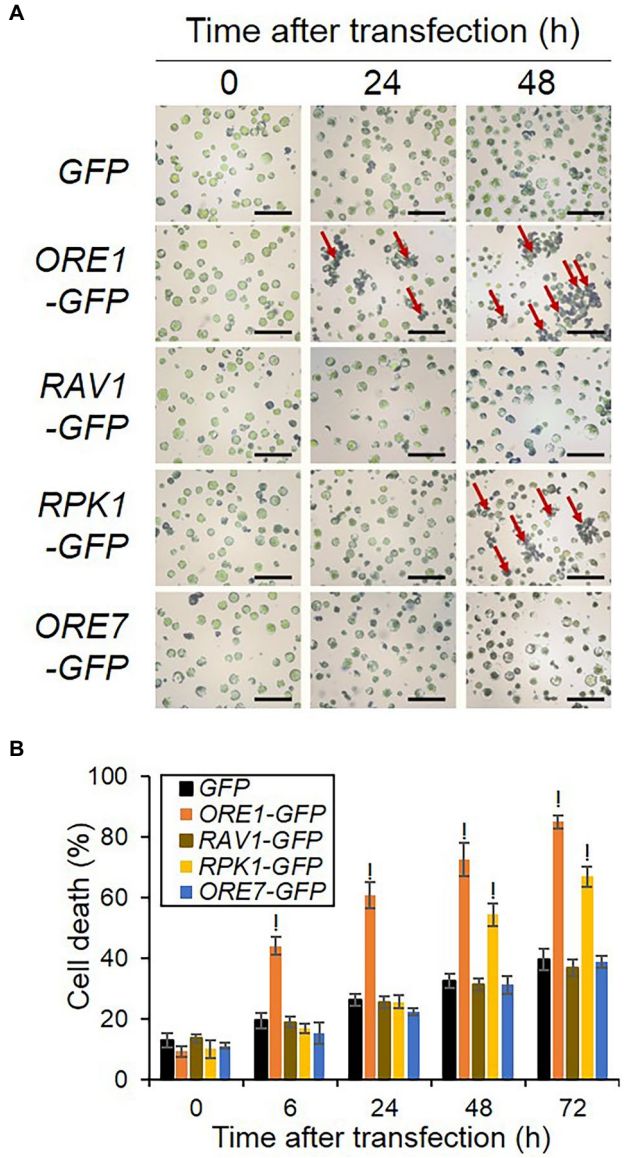

FIGURE 4 | Kinetic cell death responses induced by overexpression of various senescence regulators in Arabidopsis protoplasts. **(A)** Representative images of protoplasts stained by Evans blue. **(B)** Time-series measurement of cell death in protoplasts expressing various senescence regulators. Protoplasts were transfected with empty *GFP* (Control), *ORE1-GFP*, or *RPK1-GFP* and stained with Evans blue dye at 0, 6, 24, 48, and 72 h post-transfection. Normal and stained cells were counted in five to 12 fields containing at least 50 cells from each sample under microscopy. The percentage of dead cells was measured as [stained cells/(stained + normal cells)] × 100%. Bars = 1 mm. Data represent mean ± SE ($n = 3$). A statistical analysis was performed using a two-tailed Student's *t*-test ($^{\dagger}p < 0.0001$). Similar results were obtained in two independent trials.

RPK1 and *GFP* control induced detectable cell death at 2 DAI and 3 DAI, respectively (**Figure 5A**). Similarly, DAB-mediated H_2O_2 detection with brown staining revealed earlier and higher accumulation of H_2O_2 at 1 DAI in *ORE1*-expressed leaves only, and 2 DAI in both *ORE1*- and *RPK1*-expressed leaves, compared with those of GFP-expressed leaves (**Figure 5B**). However, superoxide staining with NBT produced a higher level of blue staining in leaves expressing *RPK1* than *ORE1* in 1 DAI, although *ORE1*-expressed leaves had higher stains than those of control at 2 DAI (**Figure 5C**). Collectively, these results indicate that *ORE1* and *RPK1* might be involved in acute cell death and superoxide-dependent intermediate cell death during leaf senescence, respectively.

DISCUSSION

Leaf senescence is the final developmental phase with self-disposal, yet it is essential for energy recycling in other organs. Although plants coordinate their leaf development, leaf senescence begins with cell-autonomous determination by multiple genetic factors (Thomas et al., 2003). The protoplast-based transient expression system is a cell-based functional assay technique that is efficient and adaptable for studying various plant developmental and physiological responses (Yoo et al., 2007; Lehmann et al., 2020). In this study, we demonstrated the potential utility of a transient gene expression system using Arabidopsis mesophyll protoplasts to assess the functional role of genes in senescence regulation in plants. We used two *SEN4*- and *SAG12-LUC* reporters for kinetic senescence response assay and validated the feasibility of functional assessment of genes in senescence regulation using their overexpression and knockdown approaches (**Figures 2, 3**). Furthermore, we investigated the functional difference between *ORE1* and *RPK1* in cell death-mediated senescence by combining the kinetic responses of the reporter assay and histochemical assay (**Figures 2, 4, 5**).

Evaluation of Senescence Response Assay Using Arabidopsis Mesophyll Protoplasts

To dissect senescence responses in protoplasts, we established two reporter sets of *SEN4*- and *SAG12-LUC* for analyzing early and late kinetic responsiveness (**Figure 1**). *SEN4* and *SAG12* were identified as transcriptionally upregulated SAGs with different kinetic profiles and have been used as molecular markers for senescence responses (Noh and Amasino, 1999; Woo et al., 2002). However, the expression pattern of *SEN4-LUC* and *SAG12-LUC* reporters in transfected protoplasts exhibited rapid induction followed by a decline, which is different from that observed in intact plants. These patterns could be attributed to the strong basal expression of many transfected plasmids and the reduction of reporter expression due to accumulating cell death over the assay. Also, we tested the possible usage of other downregulated SAGs as potential reporters, but their expression levels were marginal or variable in the protoplasts (**Supplementary Figure 1**). Nonetheless, we validated a potential usage of *SEN4*- and *SAG12-LUC* reporters for senescence responses in the protoplast system. Overexpression of identified positive senescence regulators, including *ORE1*, *RAV1*, and *RPK1*, as well as negative senescence regulators, including *ORE7,* affected the expression level of both *SEN4-LUC* and *SAG12-LUC* consistently with reports

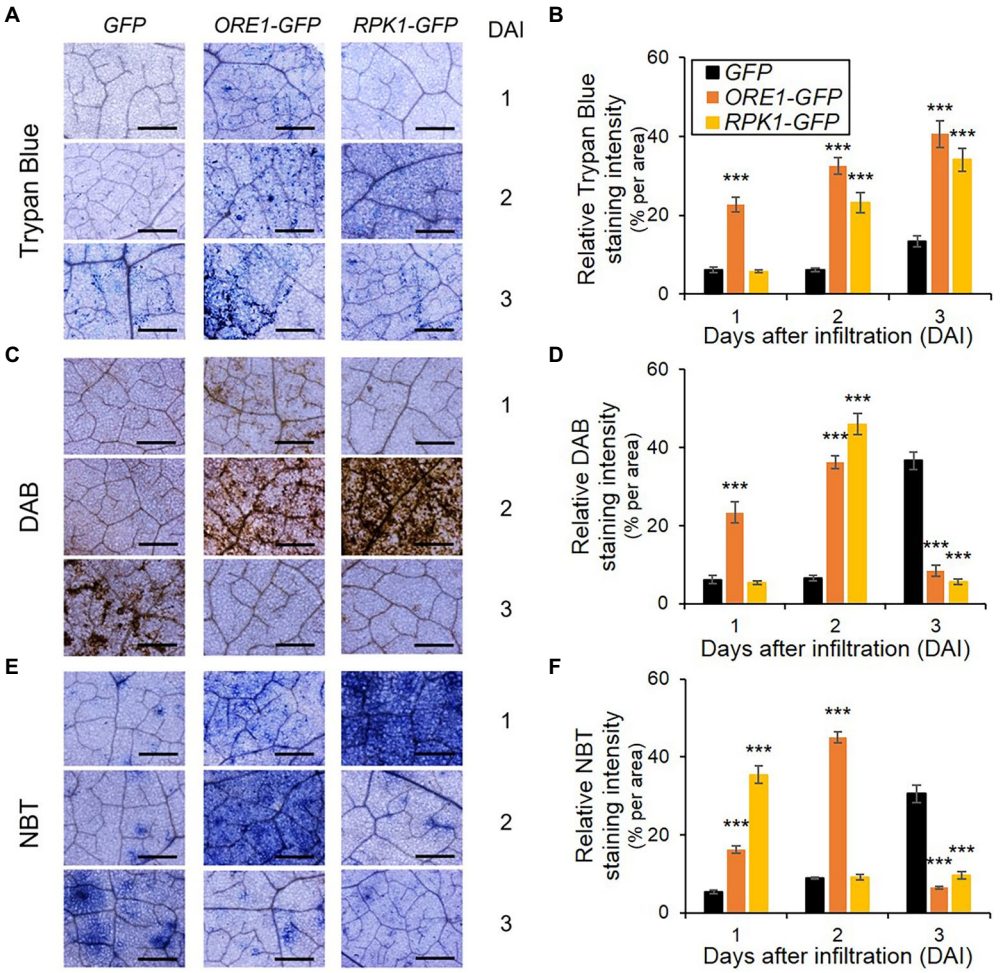

FIGURE 5 | ROS accumulation and cell death responses in *Nicotiana benthamiana* leaves expressing senescence regulators. **(A,C,E)** Representative images of leaves stained using trypan blue **(A)**, DAB **(C)**, and NBT **(E)** at indicated days after infiltration (DAI). *GFP* (Control), *ORE1-GFP*, or *RPK1-GFP* were transiently overexpressed in four-week-old *N. benthamiana* leaves through Agrobacteria infiltration. Subsequently, staining was conducted using their leaves on indicated DAI. Bars = 100 mm. **(B,D,F)** Time-series quantification of staining intensity from trypan blue **(B)**, DAB **(D)**, and NBT **(F)** images. Data represent mean ± SE ($n = 5$). Statistical analysis was conducted using a two-tailed Student's *t*-test (***$p < 0.001$), and similar results were obtained in three independent trials.

using intact leaves (**Figure 2**; Lim et al., 2007b; Kim et al., 2009; Woo et al., 2010; Lee et al., 2011). Furthermore, knockdown approaches based on amiRNA technology can be used for protoplast-based senescence assay using *SAG12-LUC*. The introduction of amiRNA constructs targeting *ORE1*, *RPK1*, or *ORE9*, as well as miR164B-OX construct, together with *SAG12-LUC* resulted in reduced *SAG12* expression in the protoplasts (**Figure 3**; **Supplementary Figure 3**). These results reinforce the notion that leaf senescence occurs by cell-autonomous signals in the mature stage. However, this does not exclude the possible involvement of an early developmental signal or additional intercellular communication in senescence regulation. This might be a reason why amiRNA for *ORE4* encoding plastid ribosomal proteins failed to show any effects on *SAG12* expression. Another limitation of this approach might be the weak or marginal responsiveness of *SEN4-LUC* reporter different from that of *SAG12-LUC* when amiRNA constructs are used as effectors. It is proposed to be because the expression of transfected *SEN4-LUC* was quickly induced up to a certain level before the substantial removal of endogenous target genes by transfected amiRNAs. Alternatively, *SEN4-LUC* can be less responsive to the repression effect by anti-senescence signals before or at the beginning of senescence since a basal expression of *SEN4* should be maintained for cell elongation in leaf growth (Woo et al., 2001; Lee et al., 2018). This is also supported by a lower effect of *ORE7* overexpression in *SEN4-LUC* than *SAG12-LUC* (**Figures 2A,B**). We also noticed a potential weakness of this approach in that cell death can cause dampened or suppressed luciferase activity, which misleads the impact of transfected effectors (**Figure 4**). Nevertheless, protoplast-based senescence assays can be used for studies of leaf senescence by complementing molecular genetic approaches

based on Arabidopsis mutants or transgenic plants. As transient transfection in protoplasts can deliver multiple plasmids simultaneously, the function of multifamily genes or interaction between senescence regulators can be dissected rapidly before laborious genetic approaches. The function or effectiveness of genes in senescence responses can be analyzed or compared in differentiated and defined protoplasts extracted from normal mature leaves, which can avoid a potential misleading interpretation by indirect or malfunctional effects of genes in an early development stage. Additionally, the protoplast-based senescence assay can be applied to high-throughput analysis based on the use of a genome-wide collection of amiRNA or open reading frame (ORF) clones, or chemical libraries.

An advantage of using this system was exemplified by comparing the functional effectiveness of known senescence regulatory genes based on the timing-dependent responses of reporters. The temporal expression patterns of SAGs can reveal the timing of gene involvement from initiation to termination of senescence. Among positive senescence regulators, *ORE1* overexpression resulted in the earliest induction of both reporters at post-transfection (**Figures 2C,D**). This is consistent with the results using amiRNA approaches for *ORE1* and *RPK1* (**Figure 3D**). This indicates *ORE1* could function as a primary and crucial genetic factor for senescence initiation. This is consistent with the role of *ORE1* as the primary genetic factor in the death circuit with the trifurcate feed-forward pathway involving EIN2, *ORE1*, and miR164 (Kim et al., 2009). Interestingly, the effectiveness of *RAV1* on the expression of reporters appears later compared with other senescence regulators. Although a previous study suggested that *RAV1* is a transcription factor with a role in triggering the initiation of leaf senescence (Woo et al., 2010), it may function as an intermediate factor following the action of primary factors like ORE1. The protoplast kinetic approach could give a more informative clue in uncovering *in vivo* role of genes over the traditional phenotypic evaluation approach. Another analytic window of reporter responses is their expression pattern. *ORE7* overexpression led to completely dampened expression (*SAG12-LUC*) and shortly induced, but dampened expression (*SEN4-LUC*; **Figures 2A,B**). It is consistent with a previous report that AT-hook protein *ORE7* functions as an epigenetic regulator for leaf senescence (Lim et al., 2007b). *ORE7* might induce chromatin condensation, which blocks the transcriptional activation of *SAG12-LUC* completely, and later induction of *SEN4-LUC*. Intriguingly, *ORE1* overexpression also dampened expression of *SEN4-LUC* and *SAG-LUC*, but it followed a higher induction of both reporters at early time points (**Figures 2A,B**). This implied that *ORE1* has a different molecular function in the regulation of senescence from the *ORE7*-mediated repression of SAGs.

ORE1 and *RPK1* in the Regulation of Cell Death-Mediated Senescence

Senescence involves the gradual loss of cellular activity and ends with death. However, an increasing amount of evidence suggests that cell death processes are not only required for dismantlement and relocation of cellular macromolecules during senescence but also mediate the initiation of leaf senescence (Guiboileau et al., 2010). An advantage of using protoplasts is the easy application to envision investigating cellular biological phenotypes combined using fluorescence-based reporters or exogenous staining. Dampened levels in the luciferase-based readout can appear not only due to strong repression but also due to cell death. Therefore, we used Evans blue staining for investigating cell death responses as senescence. *ORE1* and *RPK1* overexpression enhanced the extent of cell death, although *ORE1* increased the extent of cell death much earlier time points than *RPK1* did (**Figure 4**). Interestingly, *ORE7* had little effect on the extent of cell death, indicating the dampened expression of reporters is due to the epigenetic repression of *ORE7*. Contrarily, dampened expression of reporters by *ORE1* is likely due to the early cell death of protoplasts. Earlier and rapid induction of *SEN4* and *SAG12-LUC* by ORE1 supported the early onset of cell death signals (**Figure 2**). Additionally, *RPK1* overexpression showed enhanced cell death at later incubation time points compared with *ORE1*. Early provocation of cell death by *ORE1* supports the notion that cell death signals are likely to trigger senescence responses. Furthermore, our results suggest that *ORE1* and *RPK1* share a convergent pathway leading to senescence and cell death, but through different intermediate regulatory signaling. H_2O_2 and superoxide signaling are likely involved in *ORE1* and *RPK1*-mediated cell death and senescence (**Figure 5**). Cell death induced by *ORE1* was observed at the same time with the accumulation of H_2O_2 and superoxide, but *RPK1*-mediated cell death along with H_2O_2 production followed a rapid accumulation of superoxide. It is unclear whether ROS-induced by *ORE1* is a result or cause of cell death. In the first scenario, these results indicate that *ORE1* regulates cell death directly but *RPK1* does it indirectly through superoxide. These results are consistent with previous reports: ORE1 regulates aging-induced cell death and senescence (Kim et al., 2009); RPK1, CaM4, and RbohF Trio regulate age-dependent cell death *via* the accumulation of the superoxide (Koo et al., 2017). Alternatively, given that ROS is one of the critical factors triggering cell death, *ORE1* may also be involved in ROS-mediated senescence similar to *RPK1*. However, there is no clear evidence on the interaction between *ORE1*- and *RPK1*-mediated pathways in senescence regulation. Future works will seek to resolve the molecular mechanisms underpinning the interaction between *ORE1*- and *RPK1*-mediated pathways in cell death, including the potential involvement of ROS.

Senescence Regulatory Scheme

Based on our study, we suggest a regulatory and kinetic scheme of cellular senescence program regarding how senescence regulatory genes, such as *ORE1*, *RPK1*, *RAV1*, and *ORE7,* are involved in senescence regulation, reflected by the expression of *SEN4* and *SAG12*, partially through cell death and ROS-mediated signaling (**Supplementary Figure 6**). *ORE1* and *RPK1* function as early positive senescence regulators

through cell death- and ROS-induced senescence, respectively. *RAV1* might be involved in senescence responses as a late positive senescence regulator through the different pathways from *ORE1* and *RPK1* signals. *ORE7* is an epigenetic negative regulator that plays a dual-temporal role in the regulation of SAG expression. This scheme can provide novel insights for temporal regulatory involvement of senescence genes, although mechanistic relationships among the senescence regulators are not clearly defined in this scheme. Future studies with more diverse senescence regulators under various senescence triggering conditions will reveal a more reliable and clearer map for a kinetic function of senescence regulators during leaf senescence.

Overall, our results indicate that the protoplast transient expression system based on the luciferase-based assay is an effective tool for rapid functional dissection of senescence regulators in Arabidopsis. Combining other cellular reporters or different protoplast sources will enable us to broaden the utility of our approaches for studying various senescence processes in Arabidopsis, as well as other non-model plants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PPTD and JK conceived and designed the experiments and wrote the paper. PPTD, JHK, and JK performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.818239/full#supplementary-material

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Regulation of Arabidopsis Matrix Metalloproteinases by Mitogen-Activated Protein Kinases and Their Function in Leaf Senescence

*Hongjiao Wu[1], Qi Si[1], Jianmin Liu[1], Liuyi Yang[1], Shuqun Zhang[2] and Juan Xu[1]**

[1] State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou, China,
[2] Interdisciplinary Plant Group, Division of Biochemistry, University of Missouri, Columbia, MO, United States

Leaf senescence is a developmentally programmed cell death process that is influenced by a variety of endogenous signals and environmental factors. Here, we report that MPK3 and MPK6, two Arabidopsis mitogen-activated protein kinases (MAPKs or MPKs), and their two upstream MAPK kinases (MAPKKs or MKKs), MKK4 and MKK5, are key regulators of leaf senescence. Weak induction of constitutively active MAPKKs driven by steroid-inducible promoter, which activates endogenous MPK3 and MPK6, induces leaf senescence. This gain-of-function phenotype requires functional endogenous MPK3 and MPK6. Furthermore, loss of function of both *MKK4* and *MKK5* delays leaf senescence. Expression profiling leads to the identification of matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent endopeptidases, as the downstream target genes of MPK3/MPK6 cascade. MPK3/MPK6 activation-triggered leaf senescence is associated with rapid and strong induction of *At3-MMP* and *At2-MMP*. Expression of Arabidopsis MMP genes is strongly induced during leaf senescence, qualifying them as senescence-associated genes (SAGs). In addition, either constitutive or inducible overexpression of *At3-MMP* is sufficient to trigger leaf senescence. Based on these findings, we conclude that MPK3/MPK6 MAPK cascade and *MMP* target genes further downstream are involved in regulating leaf senescence in *Arabidopsis*.

Keywords: MAPK cascade, matrix metalloproteinase, leaf senescence, *Arabidopsis*, transcription regulation

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Yongfeng Guo,
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Kundan Kumar,
Birla Institute of Technology and Science, India

***Correspondence:**
Juan Xu
xujuan@zju.edu.cn

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INTRODUCTION

Senescence at the final stage of plant growth season or life cycle is a programmed cell death process that is influenced by both endogenous developmental programs and environmental factors (Gan and Amasino, 1997; Lim et al., 2007; Woo et al., 2013). Leaf yellowing as a result of the disruption in photosynthesis and the loss of chlorophyll due to the breakdown of the structural integrity of chloroplasts is a typical phenotypic symptom of leaf senescence. This is accompanied by other dramatic changes in cellular metabolisms, such as degradation of membrane lipids, proteins, nucleic acids, and other macromolecules (Gan and Amasino, 1997; Lim et al., 2007). Nutrients

released from the senescing leaves are recycled and used by younger growing leaves, developing seeds, or storage tissues to ensure an optimal condition for the next generation or growing season (Gan and Amasino, 1997; Guo and Gan, 2005; Lim et al., 2007; Woo et al., 2016). In addition to age, the onset and progression of leaf senescence are influenced by various internal signals including abscisic acid, salicylic acid, ethylene, and jasmonate, as well as environmental stresses such as darkness, extreme high or low temperature, nutrient deficiency, oxidative stress, and pathogen infections (Gan, 2003; Lim et al., 2007). Although many genes have been identified as senescence-associated genes (*SAGs*), their exact functions in the senescence remain largely unclear (Li et al., 2012, 2014).

Mitogen-activated protein kinase (MAPK or MPK) cascades are major pathways by which extracellular stimuli are transduced into cellular responses in eukaryotic cells (Ichimura et al., 2002; Pedley and Martin, 2005; Pitzschke et al., 2009; Andreasson and Ellis, 2010; Rodriguez et al., 2010; Tena et al., 2011; Xu and Zhang, 2015; Zhang et al., 2018). A basic MAPK cascade is composed of three interconnected kinases, a MAPK (MPK), which is activated by its upstream MAPK kinase (MAPKK, MKK, or MEK), via phosphorylation of the TXY activation motif. MAPKK activity is in turn regulated by the topmost member of the module, MAPKK kinase (MAPKKK, or MEKK), via phosphorylation. A MAPKKK receives signals from receptors/sensors either directly or indirectly. In *Arabidopsis*, there are 20 MAPKs, 10 MAPKKs, and approximately 60 putative MAPKKKs (Ichimura et al., 2002). MPK3 and MPK6, two Arabidopsis MAPKs with the highest homology, share two redundant upstream MAPK kinases, MKK4 and MKK5 (Wang et al., 2007; Su et al., 2017, 2018). In plant defense, MAPKKK3 and MAPKKK5 function redundantly upstream of MKK4/MKK5-MPK3/MPK6 in plant PAMP-triggered immunity (Bi et al., 2018; Sun et al., 2018). In plant development, YDA is upstream of the MKK4/MKK5-MPK3/MPK6 module in plant inflorescence architecture and stomatal development (Bergmann et al., 2004; Wang et al., 2007; Meng et al., 2012). Several reports have revealed the important roles of MAPK signaling cascade in regulating leaf senescence. MKK9-MPK6 cascade was shown to play a positive role in regulating leaf senescence in *Arabidopsis* (Zhou et al., 2009). MPK6-WRKY6-NPR1 and MPK6-EIN2-EIN3-ORE9 modules were implicated in SA- and MeJA-induced leaf senescence, respectively (Chai et al., 2014; Zhang et al., 2016). MAPKKK18-MKK3-MPK1/2/7, another MAPK cascade, involved in ABA-triggered leaf senescence (Matsuoka et al., 2015). Recently, MKK4/MKK5-MPK1/MPK2 cascade was reported to regulate SA-induce leaf senescence via phosphorylation of NPR1 (Zhang et al., 2020). These findings suggest a complex regulation network involved the MAPK signaling network during leaf senescence.

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidase (Rawlings et al., 2010). As a well-known proteolytic enzyme in animals, MMPs are involved in extracellular matrix remodeling, cell migration, cell proliferation, adhesion, and cellular signaling by limited proteolytic processing of their substrate proteins (Sternlicht and Werb, 2001; Overall, 2002; Parks et al., 2004; Page-McCaw et al., 2007). MMPs have also been identified in plants (Pak et al., 1997). Based on their expression patterns, plant MMPs have been implicated in plant growth and development (Delorme et al., 2000; Liu et al., 2001; Frick and Schaller, 2002; Ratnaparkhe et al., 2009; Schiermeyer et al., 2009; Zimmermann et al., 2016; Das et al., 2018). Soybean *SEMP1/Gm1-MMP* was shown to be expressed only in mature leaves, suggesting a role in tissue remodeling during leaf expansion (Pak et al., 1997). In cucumber, *Cs1-MMP* was found to be associated with senescence and cell death in cotyledon development (Delorme et al., 2000). A subtilisin-like proteinase p69b was identified as a substrate of tomato *Sl-MMPs* in cell death control (Zimmermann et al., 2016). More recently, Rice *OsMMP1* was reported to play pleiotropic roles in plant development and symplastic-apoplastic transport by modulating cellulose and callose depositions (Das et al., 2018). In *Arabidopsis*, the MMP family consists of five members named *At1-MMP* to *At5-MMP* (Maidment et al., 1999). All five *At-MMP* genes display distinct tissue/organ development-specific expression patterns, suggesting differential physiological functions for each enzyme (Maidment et al., 1999; Flinn, 2008).

In this study, we found that a weak and long-lasting activation of MPK3/MPK6 after a low-level induction of the constitutively active MAPKKs upstream (MKK4, MKK5, and their tobacco ortholog NtMEK2) is sufficient to trigger leaf senescence, which is associated with *At3-MMP* and *At2-MMP* gene activation. In contrast, loss of function of both *MKK4* and *MKK5* delays leaf senescence. Arabidopsis *MMP* gene expression is strongly induced during leaf senescence, qualifying them as senescence-associated genes (*SAG*s). Over-expression of *At3-MMP*, either constitutively or under the control of an inducible promoter, is sufficient to trigger leaf senescence. Collectively, this study reveals a signaling pathway involving MPK3/MPK6 cascade and *MMP* target genes in leaf senescence in *Arabidopsis thaliana*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

After surface-sterilized and vernalization at 4°C for 3–5 days, seeds were sown in half-strength Murashige and Skoog (MS) medium with 0.45% Phytagar and grown in a growth chamber at 22°C with continuous light (80 $\mu E/m^{-2}s^{-1}$). Seven-day-old seedlings were transplanted to soil and grown at 22°C and a 14-h-light/10-h-dark cycle. Col-0 ecotype was used as the wild type. T-DNA insertion mutants including *at1-mmp* (SALK_205145C), *at2-mmp* (SM_3_5305), *at3-mmp* (SM_3_28404), *at4-mmp* (GABI_075C07), *at5-mmp* (SAIL_390_c06), *mpk3-1*(SALK_151594), *mpk6-2* (SALK_073907), *mkk7* (SM_3_21961), and *mkk9* (SAIL_60_H06) were obtained from the Arabidopsis Biological Resource Center (ABRC). The *mkk4* and *mkk5* single tilling mutants (Zhao et al., 2014) were backcrossed with Col-0 to remove the *er-105* mutant allele and then were crossed to generate a *mkk4 mkk5* double mutant (Li et al., 2018).

Transgenic plant *GVG:NtMEK2^{DD}* (abbreviated as *DD*), in which dexamethasone (DEX)-inducible promoter-driven constitutively active *NtMEK2^{DD}* transgene, was previously described (Yang et al., 2001; Ren et al., 2002). *DD mpk3* (Wang et al., 2007) and *DD mpk6* (Liu and Zhang, 2004) were generated by genetic cross between *DD* and *mpk3-1*, *DD* and *mpk6-2*, respectively. The *mmp2 mmp3* double mutant, *mmp2 mmp3 mmp5* triple mutant, *at1;2;3;4;5-mmp* pentuple mutant, and *mkk7 mkk9* double mutant were generated by genetic cross and homozygous mutant plants were identified using T-DNA border primers and gene-specific primers (listed in **Supplementary Table 1**).

To generate DEX-inducible promoter-driven *At-3MMP* construct (*GVG:At3-MMP-dHA*) and constitutive overexpression *35S:At3-MMP-dHA* construct, we amplified the full-length *At-3MMP* cDNA fragment using primers LP1/RP1 and LP2/RP2 and cloned the PCR fragment into a modified pBlueScript II vector with a double HA tag at the *3′*-end. The *3MMP-dHA* fragment was subsequently cloned into the *Xho*I/*Spe*I sites of the pTA7002 vector and a modified pBI121 vector with 35S double enhancer and *Xho*I/*Spe*I restriction sites (pBId vector), respectively. These two binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. Arabidopsis transformation was performed by the floral dip procedure (Clough and Bent, 1998), and transformants were identified by screening for hygromycin (pTA7002 vector) or kanamycin (pBId vector) resistant T1 seedlings. Independent lines with *At-3MMP* transgene induction or expression were identified based on immunoblot analysis. From these transformations, two independent lines with a single copy of T-DNA insertion (based on the 3:1 segregation of antibiotic resistance in T2 progeny) were isolated, and homozygote transgenic plants were further identified in the progeny based on segregation of antibiotic resistance.

Leaf Senescence Assays

Leaves from 4-week-old plants were used for the leaf senescence assay. For detached leaf senescence analysis, fully expanded leaves (from the fifth to eighth leaf position) were detached and their petioles were inserted into 0.6% agar plates with DEX or EtOH solvent with the adaxial side facing up. The plates were kept under light (60 $\mu E/m^{-2}s^{-1}$) at 22°C. For observation of leaf senescence in whole plants, 4-week-old soil-grown plants were sprayed with 30 μM DEX. Whole plants were photographed at indicated times after treatment.

Measurement of Chlorophyll Content and Photochemical Efficiency

Chlorophyll was extracted with 80% (v/v) acetone from detached leaves (Arnon, 1949). Chlorophyll contents were measured at 645 and 663 nm and chlorophyll concentrations were calculated as $(20.2 \times A_{645} + 8.02 \times A_{663})$/g fresh weight. Maximal quantum yield of PSII photochemistry (F_v/F_m) was determined using a PAM 2000 portable chlorophyll fluorimeter in the dark-adapted leaf samples.

RNA Extraction and Real-Time Quantitative PCR Analysis

Total RNA was extracted using Trizol reagent (Invitrogen). RNA concentration was measured using a NanoDrop (Model 2000C). After an additional ethanol precipitation and DNase treatment, 1 μg of total RNA was used for reverse transcription. Quantitative PCR was conducted using a real-time PCR machine (Eppendorf, Germany). *EF1α* was used for internal control. The relative gene expression was calculated using the double ΔCt method. The absolute copy numbers were calculated based on the standard curve for each *MMP* gene for better assessment of the potential contribution of each *MMP* gene. The primers pairs used for qPCR are listed in **Supplementary Table 1**.

Immunoblot Analysis

Total protein was extracted from leaf tissues by grinding with 3x volume of 1xSDS sample buffer [60 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS] followed by boiling for 15 min. Protein extracts for immunoblot detection of phosphorylated MAPKs were prepared by grinding with extraction buffer [100 mM HEPES, pH 7.5, 5 mM EDTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM phenylmethylsulfony fluoride (PMSF), 5 μg/mL antipain, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 10% (v/v) glycerol]. Protein concentration was determined by using the Bio-Rad protein assay kit with BSA as a standard. Total proteins (10 μg) were separated in 10% SDS-PAGE gels. At-3MMP and DD protein induction were detected by immunoblot analysis using anti-HA (Sigma, dilution 1:10,000) and anti-FLAG (Sigma, dilution 1:10,000), respectively. Activation of MPK3 and MPK6 was detected by using anti-pTEpY (Cell Signaling, dilution 1:3,500). The blots were incubated with horseradish peroxidase-conjugated goat anti-mouse (for anti-HA and anti-FLAG) or goat anti-rabbit (for anti-pTEpY) secondary antibodies. Coomassie brilliant blue staining of duplicated gels was used to confirm equal loading.

Statistical Analysis

At least two independent repetitions were performed for experiment with multiple time points. For single time point experiments, at least three independent repetitions were done. Results from one of the independent repeats that gave similar results were shown. Two-way ANOVA assay was used to determine whether the difference between two groups of data at a specific time point is statistically significant ($P < 0.05$). Statistically different data groups are indicated by using asterisks placed above the columns in the graphs.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *At1-MMP* (At4G16640), *At2-MMP* (At1G70170), *At3-MMP* (At1G24140), *At4-MMP* (At2G45040), *At5-MMP* (At1G59970), *MPK3* (At3G45640), *MPK6* (At2G43790), *MKK4* (At1G51660), *MKK5* (At3G21220), *EF1α* (At5G60390, and *SAG12* (At5G45890).

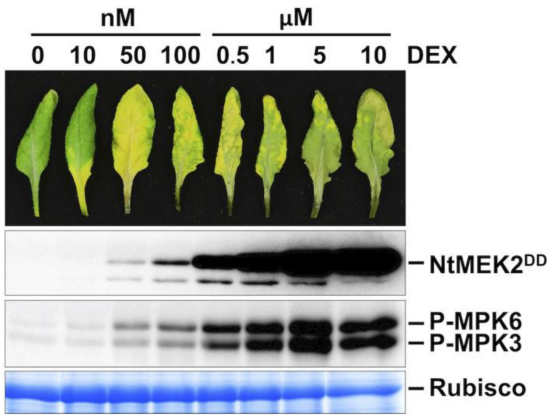

FIGURE 1 | Weak induction of constitutively active MAPKK promotes leaf senescence. Treatment of *DD* plants with different concentrations of DEX can control the amplitude of MPK3/MPK6 phosphorylation/activation. Fully expanded leaves from 4-week-old soil-grown plants were detached and their petioles were inserted into 0.6% agar medium with different concentrations of DEX. Photos were taken at 3 days after DEX treatment (first panel). Leaves treated with DEX at 0.5 μM or above showed rapid cell death and stayed green. Leaf samples were collected at 1 day after DEX treatment. DD protein induction was detected by immunoblot assay using an anti-flag antibody (second panel). MAPK activation was detected by immunoblot assay using an anti-pTEpY antibody (third panel). Coomassie brilliant blue staining of a duplicated gel was used to confirm equal loading (fourth panel).

RESULTS

Weak Induction of Constitutively Active MAPK Kinases Leads to MPK3- and MPK6-Dependent Leaf Senescence

To understand the function of MPK3 and MPK6 in *Arabidopsis* and their orthologs in other plant species, we previously generated conditional gain-of-function systems by expressing the constitutively active MAPKK (Arabidopsis MKK4 and MKK5, and tobacco NtMEK2) variants under the control of a steroid-inducible promoter (Yang et al., 2001; Ren et al., 2002). In this study, *DD* plants refer specifically to *GVG:NtMEK2^{DD}* transgenic Arabidopsis (abbreviated as *DD* for the substitution of two Ser/Thr residues in the activation loop of MAPKK with Asp to make it constitutively active). Full induction of transgene expression after dexamethasone (DEX) treatment induces high-level activation of endogenous MPK3/MPK6 in *Arabidopsis*, which triggers a hypersensitive response (HR)-like cell death (**Figure 1**; Yang et al., 2001; Ren et al., 2002). However, when these plants were treated with lower concentrations of DEX (equal or less than 100 nM), the plants showed a leaf-yellowing phenotype similar to senescence (**Figure 1**). Similarly, lower concentrations of DEX treatment of *GVG:AtMKK4^{DD}* and *GVG:AtMKK5^{DD}* Arabidopsis plants also lead to leaf-yellowing phenotype (**Supplementary Figure 1**). This phenotype is dependent on functional *MPK3* and *MPK6*. As shown in **Figure 2A**, in either *mpk3* or *mpk6* single mutant background, the gain-of-function *DD*-induced leaf yellowing phenotype was attenuated.

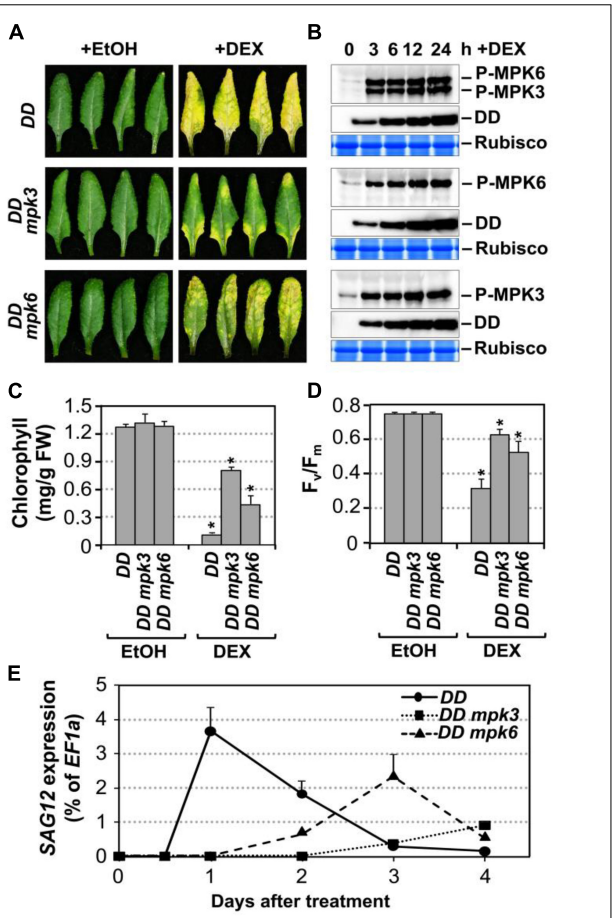

FIGURE 2 | Leaf senescence in gain-of-function *DD* plant is dependent on endogenous MPK3 and MPK6. **(A)** Fully expanded leaves from 4-week-old *DD*, *DD mpk3*, *DD mpk6* plants were detached and their petioles were inserted into 0.6% agar medium with DEX (100 nM) or EtOH (solvent control). Photos were taken 4 days after treatment. **(B)** MAPK activation (top) and DD protein induction (middle) in *DD*, *DD mpk3*, and *DD mpk6* plants after DEX treatment were detected by immunoblot analysis using anti-pTEpY and anti-Flag antibody, respectively. Coomassie brilliant blue staining of duplicated gels were used to confirm equal loading (bottom). **(C,D)** Measurement of chlorophyll content **(C)** and F_v/F_m **(D)** in leaves shown in **(A)**. Asterisks indicate a significant difference ($P \leq 0.001$). Values are means ± SD, $n = 10$. **(E)** RT-qPCR analysis of *SAG12* expression in leaves at indicated times after DEX treatment. Gene expression was calculated using the double ΔCt method and *EF1α* was used as a reference. Values were means ± SD, $n = 3$.

To confirm that the leaf yellowing phenotype in *DD* plants treated with low concentrations of DEX is indeed a senescence process, we measured typical senescence-associated physiological markers including chlorophyll content, photochemical efficiency of photosystem II (monitored as F_v/F_m), and the transcript levels of *SAG12*, a widely used molecular marker for leaf senescence (Noh and Amasino, 1999; Pontier et al., 1999). As shown in **Figure 2C**, the chlorophyll content decreased by approximately 92, 42, and 66% in the *DD*, *DD mpk3*, and *DD mpk6* plants, respectively. Similarly, F_v/F_m value reduced by ∼56% in the *DD* plants at 4 days after DEX treatment. In contrast, in *mpk3* or *mpk6* mutant background, *DD*-induced F_v/F_m reduction

was significantly attenuated (**Figure 2D**). Furthermore, *SAG12* expression was strongly induced in *DD* plants, while the induction of *SAG12* was partially compromised in *DD mpk3* and *DD mpk6* plants (**Figure 2E**). Taken together, these results suggested *DD*-induced leaf yellowing is a senescence process, which is dependent on functional downstream MPK3 and MPK6.

To further confirm that MPK3 and MPK6 were responsible for the induction of leaf senescence in *DD* plants, we measured the activation of MPK3/MPK6 using phospho-specific pTEpY antibody in *DD*, *DD mpk3*, and *DD mpk6* plants. Immunoblot analysis using anti-flag antibody showed comparable DD protein induction in all three genotypes (**Figure 2B**). The delayed leaf senescence in *DD mpk3* and *DD mpk6* plants demonstrated that leaf senescence induction by *DD* required the activation of endogenous MPK3 and MPK6. The senescence phenotype in *DD mpk3* leaves was much less severe than that in *DD mpk6* leaves in comparison to the *DD* control, suggesting that MPK3 plays a more important role than MPK6 in this process.

Leaf Senescence Was Delayed in *mkk4 mkk5* Double Mutant

Arabidopsis MKK4 and MKK5 function redundantly upstream of MPK3/MPK6 in several developmental processes (Xu and Zhang, 2015). In addition, expression of gain-of-function constitutively active DD form of these MAPKKs is sufficient to induce senescence. To gain loss-of-function evidence, we compared the leaf senescence in wild type, *mkk4* single, *mkk5* single, and *mkk4 mkk5* double mutants in detached leaves under continuous light. Slight delay in leaf senescence was observed in *mkk4* or *mkk5* single mutant compared to wild type (**Supplementary Figure 2A**). In contrast, leaf senescence was significantly delayed in the *mkk4 mkk5* double mutant (**Figure 3A**). Associated with the delayed phenotype, no induction of *SAG12* gene expression was detected in *mkk4 mkk5* double mutants 6 days after detachment. In wild type, the level of *SAG12* transcript was detected in 4 days after detachment and increased greatly afterward (**Figure 3B**). Measurement of chlorophyll content and F_v/F_m showed that greater losses in chlorophyll content and F_v/F_m value in the wild type plants than those in the *mkk4 mkk5* double mutant plants (**Figures 3C,D**). To further confirm whether MKK4 and MKK5 acted upstream of MPK3 and MPK6 during senescence, we evaluated the phosphorylation activation of MPK3 and MPK6 during different leaf senescence stage. As shown in **Figure 3E**, age-dependent leaf senescence-induced MPK3/MPK6 activation was impaired in the *mkk4 mkk5* double mutant. These data suggested that MKK4 and MKK5 are involved in plant leaf senescence and play redundant function in activating MPK3/MPK6 during leaf senescence.

In addition to MKK4 and MKK5, MKK7 and MKK9 were also reported to be upstream of MPK3/MPK6 (Xu et al., 2008; Zhou et al., 2009; Jia et al., 2016). As a result, we also examined the leaf senescence in *mkk7* single, *mkk9* single, and *mkk7 mkk9* double mutants under the same experimental condition. As shown in **Supplementary Figure 2A**, we did not see an obvious difference in leaf senescence in comparison to wild type. As a result, we conclude that MPK4/MPK5, but not MKK7/MKK9, are upstream of the MPK3/MPK6 in plant leaf senescence.

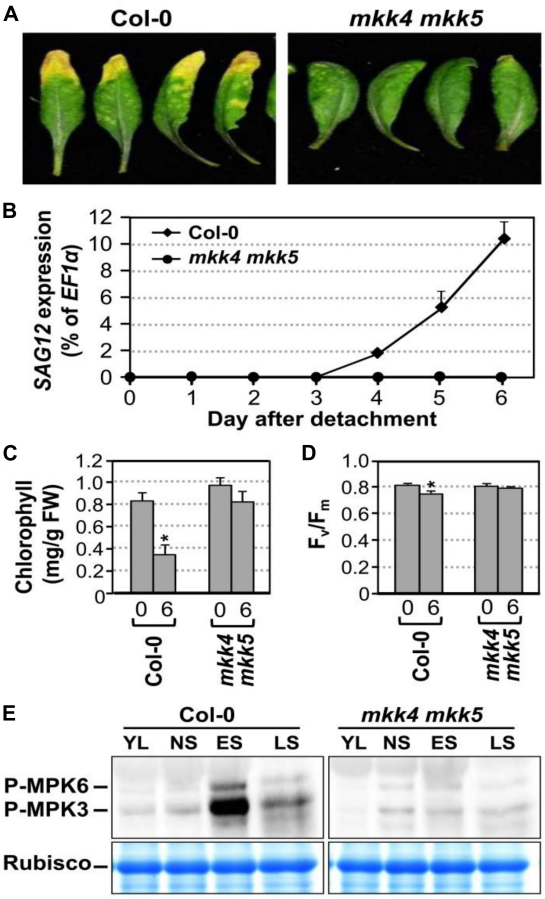

FIGURE 3 | MKK4 and MKK5 play redundant function in leaf senescence. **(A)** Leaf senescence phenotype of detached leaves of wild type and *mkk4 mkk5* double mutants. Fully expanded leaves from 4-week-old soil-grown plants were detached and their petioles were inserted to 0.6% agar medium under continuous light. Photos were taken 6 days after detachment. **(B)** RT-qPCR analysis of the transcript levels of *SAG12* in wild type and *mkk4 mkk5* double mutants at indicated times. The expression of *EF1α* was used as an internal reference. Values are means ± SD, $n = 3$. **(C,D)** Quantitative analysis of chlorophyll contents **(C)** and F_v/F_m **(D)** in leaves shown in **(A)**. Values are means ± SD, $n = 10$. **(E)** Phosphorylation/activation of MPK3/MPK6 induced by age-dependent leaf senescence was detected by immunoblot analysis using anti-pTEpY antibody (upper). Equal loading of proteins was confirmed by Coomassie brilliant blue staining of Rubisco (lower). YL, young leaves; NS, fully expanded no senescence mature leaves; ES, early senescence leaves, with < 25% leaf yellowing; LS, lately senescence leaves, with > 50% leaf yellowing (please also see **Figure 5A**). Asterisks indicate a significant difference ($P \leq 0.01$).

Expression of Matrix Metalloproteinases Genes Are Highly Induced After Gain-of-Function Activation of MPK3/MPK6 and During Senescence

To identify unknown components downstream of MPK3/MPK6 cascade in leaf senescence, we mined the expression profiling

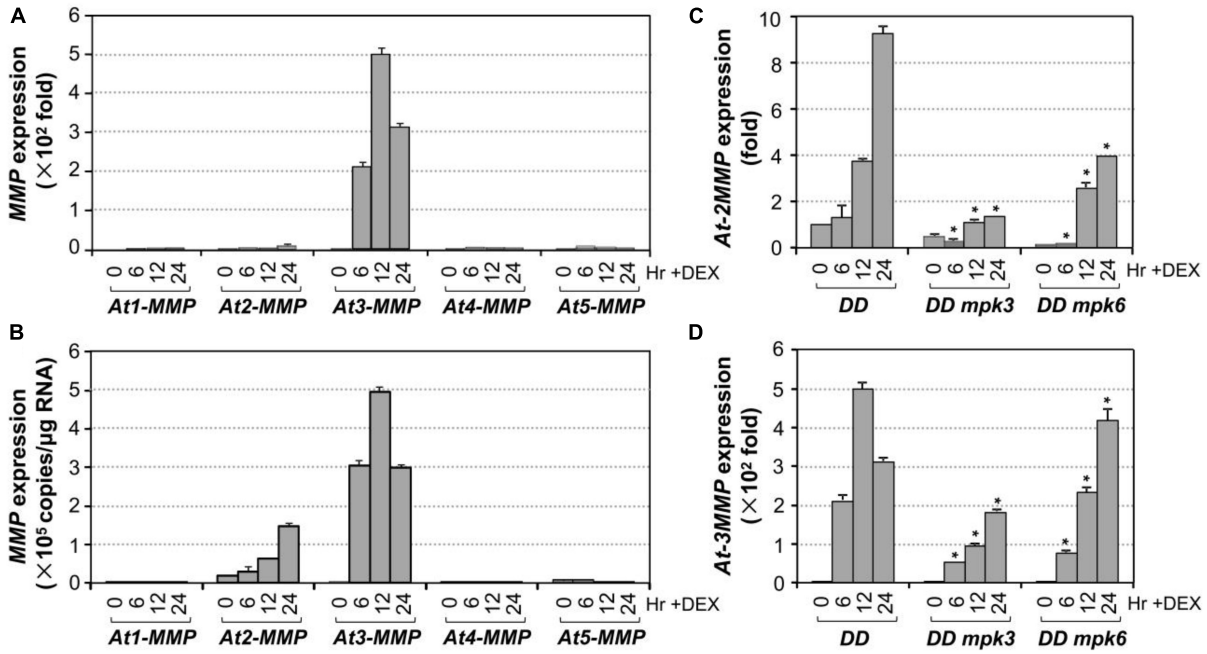

FIGURE 4 | Induction of Arabidopsis *MMP* genes expression after MPK3/MPK6 activation in the gain-of-function *DD* seedlings. **(A)** The induction of *MMP* gene expression was quantified by RT-qPCR. *MMP* induction (fold of induction relative to the level before induction) was calculated using the double ΔCt method. The expression of *EF1α* was used to normalize the samples. **(B)** Absolute expression levels of *MMP* induction was calculated as copies numbers per μg of total RNA by the calibration curve established for each *MMP* gene, which allows comparison to expression levels between different *MMP* genes. Values were means ± SD, $n = 3$. **(C,D)** Activation of *At2-MMP* **(C)** and *At3-MMP* **(D)** expression in the *DD* seedlings were compromised in either *mpk3* or *mpk6* mutant background. Gene expression was quantified by RT-qPCR and calculated as in **(A)**. Asterisks indicate a significant difference ($P \leq 0.001$). Values were means ± SD, $n = 3$.

data in *DD* transgenic plants after DEX treatment in our previous study (Su et al., 2018). *At3-MMP* is one of the highest induced genes. As a result, we quantified the expression of all five *At-MMP* genes in *DD* plants after DEX treatment and in leaves during senescence using RT-qPCR. As shown in **Figure 4A**, the expression of *At2-MMP* and *At3-MMP* was induced approximately 10 and 500-folds over its basal level, respectively. In contrast, *At1-MMP*, *At4-MMP*, and *At5-MMP* transcripts could be reliably detected, but were not induced after MPK3/MPK6 activation. To eliminate the influence of amplification efficiency during qPCR and better compare the expression levels of different *MMP* genes, we also generated the standard curves and calculated the absolute expression levels of each *MMP* after activation of *DD*. As shown in **Figure 4B**, we found that the expression levels of *At2-MMP* and *At3-MMP* were among the highest, while *At1-MMP*, *At4-MMP*, and *At5-MMP* were expressed at low levels. To confirm that MPK3 and MPK6 were responsible for the induction of *At2-MMP* and *At3-MMP* expression level in the *DD* plants, we also examined the expression of *At2-MMP* and *At3-MMP* in *DD*, *DD mpk3*, and *DD mpk6* plants. As shown in **Figures 4C,D**, while *At2-MMP* and *At3-MMP* expression was highly induced in *DD* plants after DEX treatment, their expression in *DD mpk3* and *DD mpk6* plants was partially compromised, correlating with the delayed leaf senescence (**Figure 2A**). This finding demonstrated that the induction of *At2-MMP* and *At3-MMP* in *DD* seedlings after DEX treatment is a result of MPK3/MPK6 activation.

To assess whether *MMP* genes are involved in regulating leaf senescence, we first investigated their expression patterns during the senescence process (**Figure 5A**). RT-qPCR analysis revealed that the levels of all *MMPs* except *At5-MMP* transcripts increased during the progression of leaf development and senescence (**Figures 5B–F**). *At3-MMP* had the highest copy numbers, approximately 10 times more than the other four homologs during senescence (**Figure 5D**). Based on these results, we conclude that Arabidopsis *MMPs* are senescence-associated genes (*SAGs*) and might be involved in regulating leaf senescence downstream of MPK3/MPK6 cascade.

Both Constitutive and Inducible Overexpression of *At-3MMP* Accelerates Leaf Senescence

Next, we tested whether an elevated expression of *At3-MMP* is sufficient to accelerate leaf senescence. Transgenic plants overexpressing a full-length *At3-MMP* gene with a C-terminal double HA tag driven by the constitutive 35S promoter (*35S:At3-MMP-dHA*) were generated. Two independent transgenic lines (#15 and #131) with different expression levels based on immunoblot analysis were selected for further analyses. We found that *35S:At3-MMP-dHA* plants exhibited an early senescence phenotype compared with Col-0 (**Supplementary Figure 3A**). Next, we characterized the senescence of single leaves at different ages. As shown in **Supplementary Figure 3B**, the

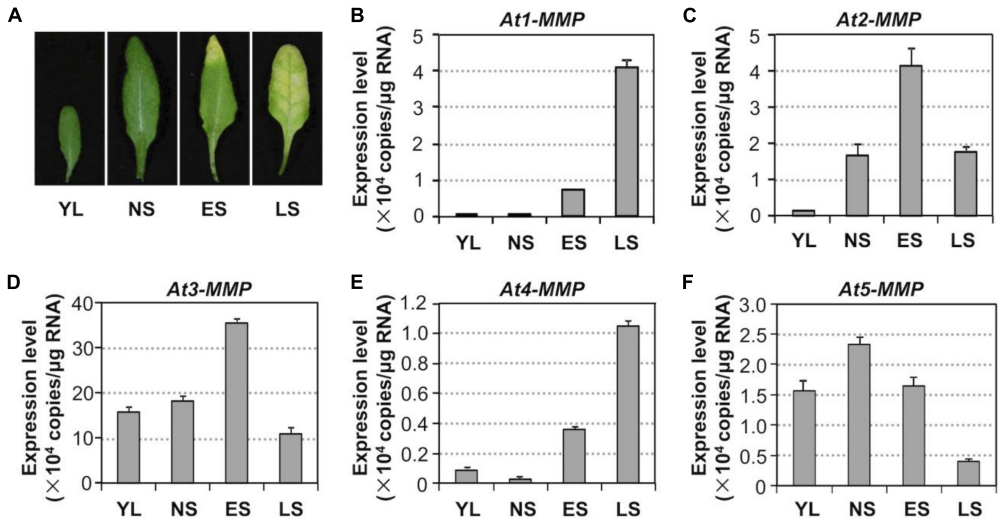

FIGURE 5 | Activation of *MMP* expression during leaf senescence in *Arabidopsis*. **(A)** Leaf senescence in wild type plants at four different developmental stages. YL, young leaves; NS, fully expanded no senescence mature leaves; ES, early senescence leaves, with < 25% leaf yellowing; LS, lately senescence leaves, with > 50% leaf yellowing. **(B–F)** Transcript levels of *MMPs* increase in an age-dependent manner. Samples were collected from leaves shown in **(A)** at the indicated developmental stages. Gene expression was quantified by RT-qPCR and calculated as copy numbers per μg of total RNA. Values are means ± SD, $n = 3$.

oldest seven leaves (numbered from the bottom; the first leaf is the oldest and the 12th leaf is the youngest) of the *35S:At3-MMP-dHA* plants showed senescence, while only three leaves of the wild-type plants senesced. In addition, the cauline leaves of *35S:At3-MMP-dHA* plants turned yellow earlier than the wild-type counterparts (**Supplementary Figure 3C**). Furthermore, there was a good correlation between the expression levels of At-3MMP protein and the severity of leaf senescence in different transgene lines (**Supplementary Figures 4A,B**). Clear acceleration of leaf senescence was also observed when the fully expanded leaves were excised from *35S:At3-MMP-dHA* plants (**Figure 6A**). We also monitored the expression of *SAG12*, chlorophyll content, and F_v/F_m. As shown in **Figure 6B**, quicker/earlier *SAG12* induction was associated with the early senescence symptoms in *35S:At3-MMP-dHA* plants. The *SAG12* transcript levels were induced significantly from 2 days in the *35S:At3-MMP-dHA* plants. In contrast, the induction of *SAG12* expression was undetectable in the first 4 days in wild type and only increased slightly afterward. Similarly, more severe reduction in chlorophyll content and F_v/F_m value happened in *35S:At3-MMP-dHA* plants (**Figures 6C,D**). Taken together, these results suggested that constitutive overexpression of *At-3MMP* is sufficient to lead to early leaf senescence.

To further confirm the role of Arabidopsis *MMP*s in leaf senescence and avoid possible secondary complications associated with constitutive overexpression, we also performed a gain-of-function study using the glucocorticoid-inducible system to direct the expression of *3MMP-dHA* transgene (*GVG:At3-MMP-dHA*). Two independent transgenic lines (#2 and #27) that accumulated At-3MMP protein at different levels were identified by immunoblot analysis and the T3 homozygous plants were selected for further experiments. We induced *At-3MMP* expression by treating 4-week-old plants with 30 μM DEX. As shown in **Figure 7A**, obvious leaf yellowing and leaf death were observed in the *GVG: At3-MMP-dHA* transgenic line #2 and #27 at 5 and 10 days, respectively. In contrast, no leaf senescence was observed in the wild type plants. No leaf senescence was detected in EtOH-treated control plants either. Associated with the leaf yellowing, chlorophyll and F_v/F_m loss and *SAG12* gene expression were readily detectable in DEX-treated *GVG: At3-MMP-dHA* plants, but not solvent control-treated plants or wild type plants (**Figures 7B–D**). Immunoblot analysis showed that At3-MMP protein level was higher in line #2 compared to line #27 (**Figure 7E**). Correlating with the induction level, line #2 leaves became fully senescent or died, while line #27 leaves were still partially green (**Figure 7A**). Taken together, overexpression of *At-3MMP*, either constitutively under the control of 35S promoter or conditionally under the control of a steroid-inducible promoter, is sufficient to promote leaf senescence.

High-Order *mmp* Mutant Fails to Show Delayed Leaf Senescence

To provide loss-of-functional evidence to support the role of *At-MMPs* in leaf senescence, we obtained loss-of-function T-DNA insertion mutants of all five Arabidopsis *MMP* genes from ABRC. Semi-quantitative RT-PCR analyses showed that *At-MMPs* transcripts were not detectable in the senescing leaves of the mutants, suggesting that these lines were all null knockout mutants (**Supplementary Figure 5**). No obvious growth/developmental or senescence phenotype was observed in *at1-mmp* to *at5-mmp* single mutant, *mmp2 mmp3*, *mmp3 mmp5* double mutants, and *mmp2 mmp3 mmp5* triple mutants (**Supplementary Figure 6**). Phylogenetic analysis revealed that all five Arabidopsis *MMP* genes shared high homology (**Supplementary Figure 7**). As a result, we generated pentuple *at1;2;3;4;5-mmp* mutant. Under our growth conditions, the

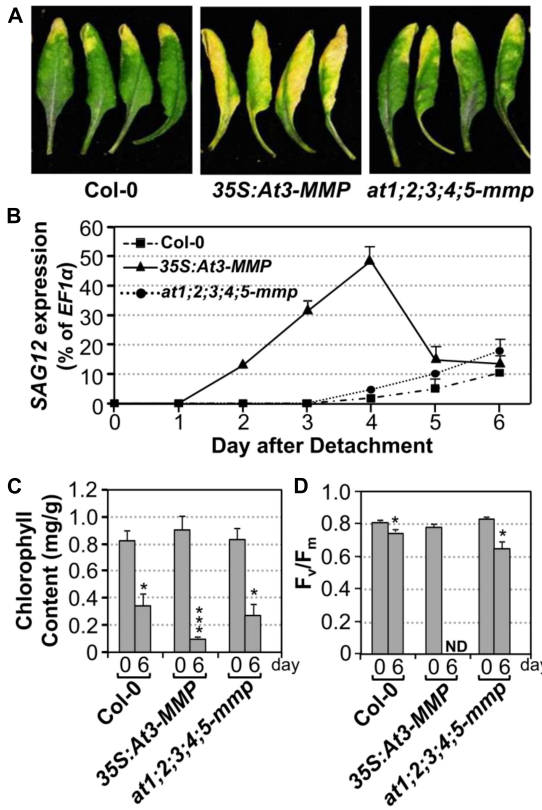

FIGURE 6 | Constitutive overexpression of *At3-MMP* leads to leaf senescence under continuous light. **(A)** Fully expanded leaves from 4-week-old soil-grown plants were detached and their petioles were inserted into 0.6% agar medium. Photos were taken after detachment 6 days. **(B)** RT-qPCR analysis of the transcript levels of *SAG12* in the leaves of plants shown in **(A)** at indicated times. The expression of *EF1α* was used as an internal reference. Values are means ± SD, $n = 3$. **(C,D)** Chlorophyll content **(C)** and F_v/F_m **(D)** in leaves shown in **(A)**. Values are means ± SD, $n = 10$. ND, not detectable. Asterisks indicate a significant difference (* $P < 0.05$, *** $P \leq 0.001$).

at1;2;3;4;5-mmp high-order mutant was still wild-type like and no delayed senescence was observed (**Supplementary Figure 6**). We also examined the senescence of detached leaves of wild type and *at1;2;3;4;5-mmp* higher-order mutant under continuous light and found that there was no obvious difference between them in leaf yellowing, *SAG12* gene activation, chlorophyll content, and F_v/F_m (**Figure 6**). Taken together, we conclude that although expression of *At3-MMP* is sufficient to induce leaf senescence, loss of *MMP*s is not sufficient to block leaf senescence, suggesting the presence of other redundant pathway(s) that are sufficient to signal/execute leaf senescence in the absence of *MMPs*.

DISCUSSION

In this study, we demonstrate that MPK3/MPK6, and their upstream MAPKKs, MKK4, and MKK5, are involved in regulating leaf senescence in *Arabidopsis* (**Figures 1–3**). Four of the five MMP family members are highly induced during leaf senescence (**Figure 5**), qualifying them as senescence-associated genes (*SAGs*). Among them, *At3-MMP* is induced to the highest level. Interestingly, *At3-MMP* is one of the top differentially expressed genes in *DD* plants (Su et al., 2018). More detailed qPCR analyses demonstrated that the expression of *At-3MMP* and, to a lesser extent, *At-2MMP* is rapidly induced by MPK3/MPK6 activation (**Figure 4**). Either constitutive or inducible overexpression of *At3-MMP* is sufficient to trigger leaf senescence (**Figures 6**, **7** and **Supplementary Figure 3**), suggesting a linear pathway from MPK3/MPK6 MAPK cascade to *MMP* target genes in leaf senescence.

Arabidopsis MPK6, along with MKK9, a MAPKK, has been implicated in leaf senescence in Arabidopsis (Zhou et al., 2009). It was reported that senescence is delayed in detached leaves of both *mpk6* and *mkk9* single mutant plants, while overexpression of *MKK9* accelerates premature senescence in leaves. In this study, we tested both *mpk3* and *mpk6* single mutants. As shown in **Supplementary Figure 2A**, neither showed a clear senescence phenotype. We failed to observe any senescence phenotype in the chemical genetically rescued *MPK3SR* and *MPK6SR* double mutant either (**Supplementary Figure 2B**). The genotypes of *MPK3SR* and *MPK6SR* double mutant plants are *mpk3 mpk6 proMPK3:MPK3*^{TG} and *mpk3 mpk6 proMPK6:MPK6*^{YG}, respectively. In the presence of 4-amino-1-tert-butyl-3-(1′-naphthyl) pyrazolo [3,4-d] pyrimidine (NA-PP1), the activity of chemical-sensitized MPK3^{TG} or MPK6^{YG} is inhibited, making the *MPK3SR* and *MPK6SR* activity null *mpk3 mpk6* double mutants (Xu et al., 2014; Su et al., 2017). The failure in observation of any senescence phenotype of *MPK3SR* and *MPK6SR* double mutant plants is likely because NA-PP1 can be metabolized in plant cells rather quickly, which makes it difficult to maintain a MPK3/MPK6 activity null condition for an extended period of time (over several days) to inhibit senescence. In contrast, we consistently observed a delayed senescence in *mkk4 mkk5* double mutant leaves (**Figure 3**). In this double mutant, the *mkk4* tilling allele has a substitution of the conserved Pro residue at the 240 position of the catalytic domain by Ser residue (CCT to TCT) and the mutated kinase carries ∼10% of the residual kinase activity. The *mkk5* tilling allele is a null mutant with a premature stop codon (Arg72 to opal; CGA to TGA). As a result, the *mkk4 mkk5* double mutant has about 5% of the residual activity if MKK4 and MKK5 contribute equally to activate downstream MPK3/MPK6 (Su et al., 2017). In addition to Arabidopsis MKK4 and MKK5 (two plant Group C MAPKKs), Arabidopsis MKK7 and MKK9, two members sharing closest homolog in the Group D MAPKKs (Ichimura et al., 2002), were also reported to be upstream of MPK3 and/or MPK6 (Xu et al., 2008; Jia et al., 2016). However, we did not observe any obvious senescence phenotype in *mkk7* single, *mkk9* single, and *mkk7 mkk9* double mutants (**Supplementary Figure 2A**). As a result, we conclude that MKK4/MKK5, but not MKK7/MKK9, are upstream of the MPK3/MPK6 in plant leaf senescence.

In the gain-of-function *DD* transgenic system, our previous study showed that *DD* transgene expression is detectable within 2 h and HR-like cell death appears ∼24 h after the application

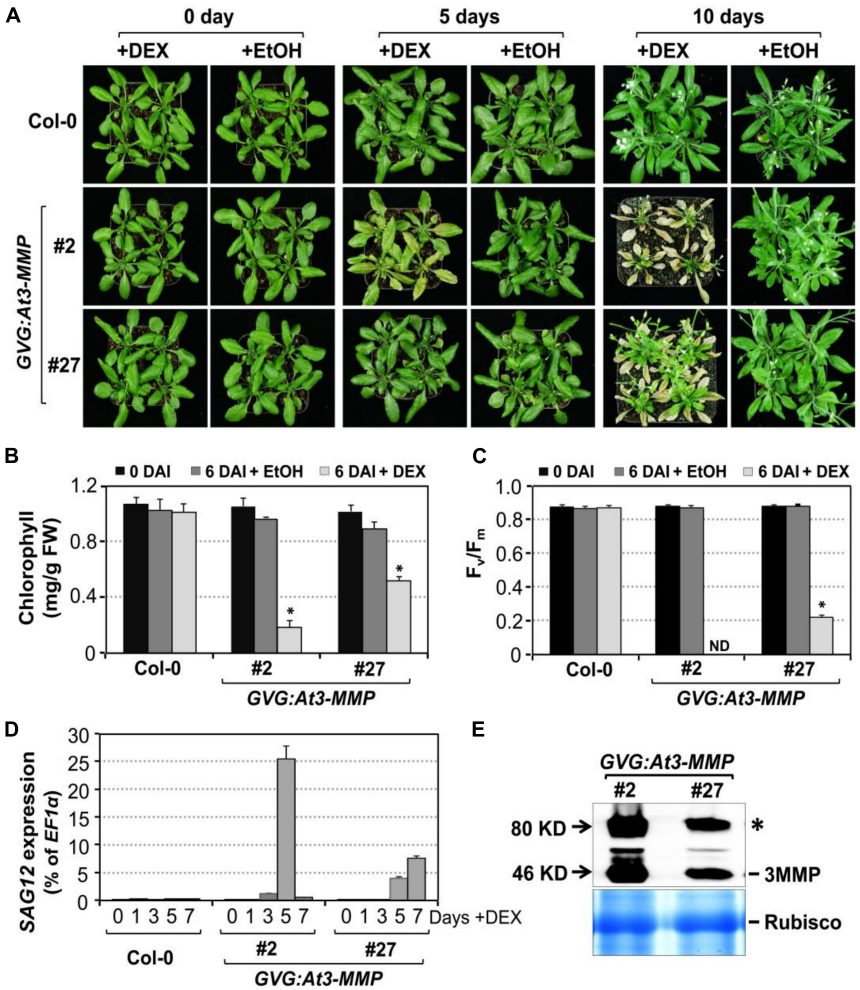

FIGURE 7 | Conditional expression of *At-3MMP* is sufficient to induce leaf senescence. **(A)** The senescence phenotype of inducible *At3-MMP* transgenic lines (#2 and #27) and wild type plants after treatment with 30 μM DEX or EtOH (solvent control) for 0, 5, and 10 days. 4-week-old soil-grown plants were sprayed with DEX or EtOH. Photos were taken at the indicated time points. **(B,C)** Chlorophyll content **(B)** and F_v/F_m **(C)** of fifth to seventh leaves in wild-type and transgenic plants before and after DEX or EtOH treatment. Values are means ± SD, *n* = 10. ND: no detected. **(D)** RT-qPCR analysis of *SAG12* transcript levels at indicated times after DEX treatment. Values are means ± SD, *n* = 3. **(E)** Induction of At3-MMP protein at 24 h after DEX (5 μM) treatment. HA-tagged At3-MMP protein was detected by immunoblot analysis using anti-HA antibody (upper). Coomassie brilliant blue staining of a duplicated gel was used to confirm equal loading (bottom). Asterisks indicate a significant difference ($P \leq 0.001$).

of DEX. The dead leaves stay green and became brittle, similar to HR cell death triggered by pathogens (Ren et al., 2002). In this study, we confirm that strong and prolonged MPK3/MPK6 activation causes HR-like cell death in *DD* leaves after treatment with higher concentrations of DEX (>100 nM). In contrast, when *DD* leaves are treated with lower concentrations of DEX (<100 nM), leaf senescence is induced, which is associated with lower levels of MPK3/MPK6 phosphorylation and activation (**Figure 1** and **Supplementary Figure 1**). These results provide another piece of evidence to support the hypothesis that MAPK signaling strength/duration could be important to the biological outcomes. Based on genetic evidence, we concluded that different developmental processes may have differential signal thresholds (strength and/or duration), which could specify the MAPK function in a quantitative way (Wang et al., 2007, 2008; Xu and Zhang, 2015).

Unlike mammalian *MMP* genes, which have been studied in great detail, the functions of *MMPs* in plants are mostly unknown. Based on the gene expression pattern, *SMEP1*, a plant matrix metalloproteinase gene isolated from soybean, was found to be expressed in mature leaves but not in young leaves and tissues (Pak et al., 1997). In cucumber, the *Cs1-MMP* was expressed in leaves during senescence and may be involved in programmed cell death (Delorme et al., 2000). In this study, we demonstrate that *At-MMPs* are *SAGs*, and play an important role in leaf senescence. Four of the five *MMP* transcripts are induced during the progression of leaf senescence, especially *At3-MMP*, which shows the greatest induction (**Figure 5**). Furthermore, constitutive or inducible overexpression of *At3-MMP* is sufficient to induce leaf senescence, as indicated by a loss/reduction in chlorophyll and photochemical efficiency, and the induction of *SAG12* gene expression (**Figures 6**, **7** and

Supplementary Figure 3). Collectively, these data suggest that plant *MMPs* are involved in regulating leaf senescence.

We obtained T-DNA insertion mutants of all five *MMP* genes from ARBC. Semi-quantitative PCR analysis confirms that all of them are complete knock-out mutants (**Supplementary Figure 5**). However, we failed to observe a difference in leaf senescence between wild type and the single, double, triple, and pentuple *mmp* mutants (**Figure 6** and **Supplementary Figure 6**), suggesting parallel pathway(s) that is sufficient to execute leaf senescence. Transcriptome analyses of leaf senescence identified thousands of *SAGs*. However, most *SAG* mutants do not have altered leaf senescence, probably due to functional redundancy or the lack of a pronounced effect on leaf senescence (He et al., 2001). A large number of plant signaling molecules including plant hormones, kinases including receptor-like protein kinases, and transcription factors contribute to the regulation and execution of senescence at the cellular and subcellular levels, suggesting functionally redundant pathways (He et al., 2001; Guo et al., 2004; Lim et al., 2007; Schippers et al., 2007; Kim H. J. et al., 2016; Kim J. et al., 2016). Recently, MAPKKK18 was identified as a positive regulator of leaf senescence. It controls the timing of leaf senescence via its kinase activity in an ABA-dependent manner (Matsuoka et al., 2015). Phylogenetic analysis revealed that MAPKKK18 belongs to a different subgroup of MAPKKKs as YDA, MAPKKK3, and MAPKKK5, three MAPKKKs that have been functionally placed upstream of the MKK4/MKK5-MPK3/MPK6 module in various biological processes (Bergmann et al., 2004; Wang et al., 2007; Meng et al., 2012; Bi et al., 2018; Sun et al., 2018; **Supplementary Figure 8**). MEKK1, a MAPKKK that belongs to yet another subgroup of MAPKKKs, was reported to regulate leaf senescence by directly phosphorylating the WRKY53, a senescence-promoting transcription factor (Miao et al., 2007). At this stage, whether MAPKKK18 and/or MEKK1 are in the same MAPK cascade as MKK4/MKK5 and MPK3/MPK6 in leaf senescence is unknown.

In *35S:At3-MMP-HA* and *GVG:At3-MMP-HA* transgenic plants, we detected two HA-tagged protein bands with molecular masses of 46 and 80 kDa by immunoblot analysis (**Figure 7** and **Supplementary Figure 4**). The theoretical mass of At3-MMP based on its amino acid sequence is ∼43 kDa. A similar finding was reported in a previous study, in which the larger protein band was speculated to be glycosylated or dimer form of MMP protein (Schiermeyer et al., 2009). Substrate identification will provide further insights into how MMPs carry out their function(s) in plant leaf senescence, an important part of plant growth/development that is influenced by a wide variety of internal and environmental factors. Leaf senescence normally occurs at the final stage of the plant growth season or life cycle, and is essential for nutrient recycling and crop yield (Gan, 2003; Lim et al., 2007). In contrast, premature leaf senescence, as an exit strategy when plants are confronted with biotic/abiotic stresses, may reduce yield in crop by limiting the growth stage and causing post-harvest spoilage. As a result, understanding the regulation of leaf senescence will not only reveal insights into this fundamental developmental process but also shed light on ways of manipulating senescence for agriculture application.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: NCBI Sequence Read Archive (SRP111959).

AUTHOR CONTRIBUTIONS

HW performed most of the experiments and analyzed the data. QS, JL, and LY provided technical assistance. HW, SZ, and JX conceived the project, designed the experiments, and wrote the manuscript. JX served as the author responsible for contact and ensure communication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.864986/full#supplementary-material

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REVIEW
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Function of Protein Kinases in Leaf Senescence of Plants

Fengbo Yang[1], Yuchen Miao[2], Yuyue Liu[2], Jose R. Botella[3], Weiqiang Li[2], Kun Li[2] and Chun-Peng Song[1]**

[1] State Key Laboratory of Crop Stress Adaptation and Improvement, School of Life Sciences, Henan University, Kaifeng, China, [2] State Key Laboratory of Cotton Biology, Henan Joint International Laboratory for Crop Multi-Omics Research, School of Life Sciences, Henan University, Kaifeng, China, [3] School of Agriculture and Food Sciences, University of Queensland, Brisbane, QLD, Australia

Leaf senescence is an evolutionarily acquired process and it is critical for plant fitness. During senescence, macromolecules and nutrients are disassembled and relocated to actively growing organs. Plant leaf senescence process can be triggered by developmental cues and environmental factors, proper regulation of this process is essential to improve crop yield. Protein kinases are enzymes that modify their substrates activities by changing the conformation, stability, and localization of those proteins, to play a crucial role in the leaf senescence process. Impressive progress has been made in understanding the role of different protein kinases in leaf senescence recently. This review focuses on the recent progresses in plant leaf senescence-related kinases. We summarize the current understanding of the function of kinases on senescence signal perception and transduction, to help us better understand how the orderly senescence degeneration process is regulated by kinases, and how the kinase functions in the intricate integration of environmental signals and leaf age information.

Keywords: leaf senescence, protein kinase, phytohormone, reactive oxygen species, calcium signal, metabolism

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Zhonghai Li,
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Chunjiang Zhou,
Hebei Normal University, China
Jan Martinec,
Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Czechia

****Correspondence:***
Kun Li
likun@henu.edu.cn
Chun-Peng Song
songcp@henu.edu.cn

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INTRODUCTION

Leaves are the main organs for photosynthesis in plants and places for water transpiration and carbon dioxide (CO_2) exchange (Efroni et al., 2010; Kalve et al., 2014; Thomas and Ougham, 2014). Leaf senescence involves the orderly breakdown of cellular structures (including chloroplasts), and hydrolysis of macromolecules (proteins, carbohydrates, lipids, and nucleic acids, etc.) to generate nutrients, which will be reallocated into developing fruits, seeds or storage tissues which depending on the life cycle of species (Rivero et al., 2007; Watanabe et al., 2013, 2018; Kim J. et al., 2016; Thomas and Ougham, 2016). Leaf senescence occurs at various levels including the deterioration and death of cells, tissues, or the whole leaf, and it is a critical process for plant survival as well as ensuring the successful reproduction of the next generation. Meanwhile, leaf senescence is a highly complex process involving orderly and highly coordinated degeneration and remobilization, which are tightly regulated by many genes, including NAC, WRKY and other transcription factors (Koyama, 2018; Woo et al., 2018; Ahmad and Guo, 2019; Jan et al., 2019).

Leaf senescence is generally controlled by plant age and developmental stage, additionally internal factors and external environmental signals also trigger the onset of senescence. The internal

factors mainly include endogenous hormones levels, source-sink relationships, and carbon-nitrogen resource allocation. The external environmental signals mainly refer to abiotic and biotic stresses, such as high salinity, low water status, soil nutrient deficiency, unfavorable light regimes, extreme temperature changes, and pathogen infection, all of them are involved in triggering the aging process of leaf. Since there are many different signals perception and transduction pathways actions coordinately control leaf senescence, it is reasonable to assume that there are significant overlaps and cross-talks between different signaling pathways in the regulation of leaf senescence, therefore, leaf senescence is regulated by a complex and precise network (Buchanan-Wollaston et al., 2003; Lim et al., 2007; Jibran et al., 2013; Woo et al., 2019).

In this review, we will summarize in-depth synopsis of protein kinases in the regulation of leaf senescence. We will present the function of protein kinases in different signal transduction pathways and regulatory networks involved in leaf senescence, to explore the molecular mechanisms of how internal and external senescence cues are perceived and ultimately lead to transcriptional level regulation of senescence associated genes and thus the execution of leaf senescence.

AGE-DEPENDENT AND STRESS-INDUCED LEAF SENESCENCE IN PLANTS

Leaf senescence is generally defined as age-dependent and the stress-induced senescence. Plants display senescence syndrome even under optimal growth conditions, which is the best known as age-dependent senescence (Buchanan-Wollaston et al., 2005). The initiation of age-dependent leaf senescence is tightly related to the developmental cues of plants, and leaves start senescence process when they reach to maturation stage, and the change of source-sink relationship may be one of the factors to trigger age-dependent leaf senescence (Thomas, 2013; Rankenberg et al., 2021). In addition, leaf senescence can also be induced by various signals, including hormonal, nutritional status, abiotic and biotic stresses during development process (Schippers, 2015; Schippers et al., 2015). Stress-induced leaf senescence only occurs in mature plants, and Arabidopsis juvenile leaves do not show senescence symptoms under ethylene treatment, and the length of vegetative growth stage is shortened under stress conditions (Jing et al., 2005; Miryeganeh, 2021).

Previous studies have found that although the initiation signals of leaf senescence are different, plant leaves show similar morphological, physiological, biochemical and transcriptional changes, which are the consequence of the similar signal transduction systems both in age-dependent and stress-induced leaf senescence (Guo et al., 2004; Guo and Gan, 2005). Meanwhile, many senescence associated genes (*SAGs*) are involved in both age-dependent senescence and stress-induced senescence (van der Graaff et al., 2006; Guo and Gan, 2012), therefore, the underlying regulatory mechanisms may overlap (Kim H. J. et al., 2016). Protein kinases, such as RPK1 and SnRK1, are also involved in both age-dependent senescence and stress-induced senescence (Lee et al., 2011; Cho et al., 2012; Kim et al., 2017; Koo et al., 2017). We mainly focus on function of protein kinases in stress-induced leaf senescence in this review.

RECEPTOR-LIKE KINASES IN LEAF SENESCENCE

Plant typical receptor-like kinases (RLKs) are transmembrane proteins typically containing an N-terminal extracellular domain for ligand binding and a C-terminal intracellular kinase domain to phosphorylate downstream components. RLKs usually form homo- or hetero-dimers (Jose et al., 2020). Ligand binding to the extracellular domain induces trans-phosphorylation of the monomers before transmitting the signal to downstream components by phosphorylation to activate the regulatory network. The Arabidopsis (*Arabidopsis thaliana*) genome contains more than 600 RLKs, including approximately 150 receptor-like cytoplasmic kinases (RLCKs) that lack the extracellular domain and associate with receptor complexes to function (Liang and Zhou, 2018). RLKs have important function in diverse biological processes, including plant growth and development, self-incompatibility, hormone perception and resistance to biotic and abiotic stresses (Berger, 2009; Chae et al., 2009; Vaid et al., 2013; Antolín-Llovera et al., 2014; Ye et al., 2017). Extracellular RLK domains are classified into more than 20 classes, including S-domains, leucine-rich repeats (LRR), epidermal growth factor-like (EGF), lectin-like, tumor-necrosis factor (TNF), and pathogenesis related-5 protein (PR5), etc. Although a large number of RLKs have been identified in plants, initial studies mainly focused on expression patterns and biochemical analyses, therefore, functional studies of their signal transduction pathways are still inadequate, and most RLKs are still "orphan," i.e., the ligands and downstream targets of most RLKs in Arabidopsis and Rice (Shiu et al., 2004), and a group of RLKs as cell surface receptors for root meristem growth factors are still unknown (Yu and Luan, 2016), which need further investigation.

Cell surface-localized RLKs can sense and transmit a variety of signals in response to environmental stresses and play essential roles in a wide range of physiological and developmental processes, including leaf senescence. The roles of a number of key RLKs in regulating leaf senescence have been characterized in various plant species (**Table 1**). A LRR-RLK from bean (*Phaseolus vulgaris*) was named senescence-associated receptor-like kinase (SARK) because its mRNA and protein levels increased during leaf senescence (Hajouj et al., 2000). PpSARK (*Physcomitrella patens* senescence-associated receptor-like kinase), with high homology to the bean SARK, is involved in the regulation of moss (*Physcomitrella patens*) senescence (Li et al., 2018). Another LRR-RLK gene involved in the regulation of leaf senescence was isolated in the soybean (*Glycine max*) and named *Glycine max* senescence-associated receptor-like kinase (GmSARK). Downregulation of *GmSARK* in transgenic soybean resulted in delayed leaf senescence while overexpressing lines showed increased senescence rates

TABLE 1 | Receptor-like kinases (RLKs) function in the regulation of leaf senescence.

Kinase name	Species	Performance during leaf senescence	Function	Role	References
PvSARK	*P. vulgaris*	mRNA and protein levels increased under natural- and induced-leaf senescence	Unknown	Unknown	Hajouj et al., 2000
PpSARK	*P. patens*	The gain-function- mutants display insensitive to ABA induced leaf senescence	Regulates high salt and ABA responses	Negative	Li et al., 2018
GmSARK	*G. max*	*GmSARK* knock-down plants show delay leaf senescence and the over-expression lines display early leaf senescence	Regulating chloroplast development and chlorophyll accumulation	Positive	Li X. et al., 2006
AtSARK	*A. thaliana*	*AtSARK*-overexpressing seedlings display precocious leaf senescence	Regulating leaf senescence through synergistic actions of auxin and ethylene	Positive	Xu et al., 2011
RPK1	*A. thaliana*	*rpk1* mutants exhibit delayed age-dependent and ABA-induced senescence symptoms	Regulates the expression of *SAGs* and ABA-inducible genes	Positive	Lee et al., 2011; Koo et al., 2017
SERK4	*A. thaliana*	*SERK4* was up-regulated during leaf senescence, and *serk4* mutants display a significant early leaf senescence	Regulates ROS generation, Ca^{2+} homeostasis and cell death	Negative	Li et al., 2019; Yu et al., 2019; Zhou et al., 2019
OsSRLK	*O. sativa*	*OsSRLK* is upregulated in senescing rice leaves. The detached leaves of *srlk* contained more green pigment during dark-induced senescence	Participates in phytohormone-mediated chlorophyll degradation under dark-induced senescence	Positive	Shin et al., 2019
LMK1	*N. benthamiana*	Response to high C/low N-nutrient stress and overexpression of *LMK1* induces cell death in *N. benthamiana* leaves	Unknown	Positive	Li X. et al., 2020
AtWAKL10	*A. thaliana*	*wakl10* mutants display earlier leaf senescence and the overexpression plants delay the aging process	Unknown	Negative	Li et al., 2021
HvLysMR1	*H. vulgare*	Transcript accumulates during leaf senescence	Unknown	Unknown	Ouelhadj et al., 2007
OsSIK2	*O. sativa*	*OsSIK2*-overexpression seedlings exhibit early leaf development and delayed dark-induced senescence, while *sik2* mutants show opposite phenotype	Enhances plants tolerance to abiotic stress	Negative	Chen et al., 2013
CRK5	*A. thaliana*	*crk5* mutants show accelerated leaf senescence	Regulates the accumulation of ROS, ethylene, SA	Negative	Burdiak et al., 2015
OsBBS1	*O. sativa*	*bbs1* seedlings are hypersensitive to salt and show premature leaf senescence	ROS accumulation and cell death	Negative	Zeng et al., 2018

(Li X. et al., 2006). AtSARK (*A. thaliana* senescence-associated receptor-like kinase), the homolog of GmSARK, positively regulates leaf senescence in Arabidopsis, with overexpression of *AtSARK* leading to premature of leaf senescence, whereas down-regulation caused delayed leaf senescence (Xu et al., 2011). In Arabidopsis, RPK1 (receptor protein kinase 1) has a positive role in age-dependent and ABA (abscisic acid)-induced leaf senescence (Lee et al., 2011), mediated by the NADPH oxidase RbohF (respiratory burst oxidase homolog protein F) (Koo et al., 2017). Another Arabidopsis LRR-RLK, SERK4 (somatic embryogenesis receptor kinase 4) which is induced during leaf senescence as well as by several abiotic stresses has a negative role in the regulation of leaf senescence (Li et al., 2019; Yu et al., 2019; Zhou et al., 2019). The rice (*Oryza sativa*) senescence-induced receptor-like kinase (OsSRLK) is involved in phytohormone-mediated chlorophyll degradation under dark-induced senescence (Shin et al., 2019). Phosphorylation levels of a leucine-rich repeat malectin kinase 1 (LMK1) were strongly affected by high C/low N-nutrient stress and overexpression of LMK1 induced cell death in *Nicotiana benthamiana* leaves (Li X. et al., 2020).

Besides LRR-RLKs, other RLKs also have roles in the regulation of leaf senescence. The WAK-like kinases (WAKLs) belong to EGF-RLKs, and one of WAKLs, AtWAKL10 is induced by ABA, JA, and SA, and it negatively modulates the leaf senescence progression, the *atwakl10* mutants display accelerated leaf senescence and *AtWAKL10* overexpression plants show opposite phenotype (Li et al., 2021). The expression of *HvLysMR1*, a barley (*Hordeum vulgare*) lysine motif RLK is induced by heavy metal and calcium ionophore A23187 treatment as well as leaf senescence (Ouelhadj et al., 2007). OsSIK2 (*O. sativa* stress-induced protein kinase gene 2), an S-domain receptor-like kinase in rice (*O. sativa*), is expressed mainly in leaf and sheath, and induced by several abiotic stresses. Transgenic rice plants over-expressing *OsSIK2* exhibited delayed dark-induced leaf senescence (Chen et al., 2013). Mutation of the Arabidopsis cysteine-rich receptor-like kinase CRK5, produce accelerated senescence correlated with accumulation of reactive oxygen species (ROS), ethylene and salicylic acid (Burdiak et al., 2015). OsBBS1/OsRLCK109 encodes a RLCK in rice, it is involved in salt stress response and leaf senescence, seedlings of *bbs1* (*bilateral blade senescence 1*) mutants are

hypersensitive to salt and show premature leaf senescence phenotype (Zeng et al., 2018).

HORMONE SIGNALING AND INTRACELLULAR SECOND MESSENGERS REGULATED PROTEIN KINASES INVOLVED IN LEAF SENESCENCE

Hormones are essential for plant development and stress responses, thus they have a significant role in the regulation of age-dependent and stress-induced leaf senescence. Ethylene, ABA, jasmonic acid, salicylic acid, brassinosteroids and strigolactone promote, while cytokinins and gibberellins inhibit leaf senescence (Jan et al., 2019; Woo et al., 2019; Chen et al., 2020). Many kinases involved in plant hormone signaling and have been associated to the regulation of leaf senescence (**Figure 1** and **Supplementary Table 1**). The Arabidopsis EDR1 (enhanced disease resistance 1), which is an mitogen-activated protein kinase kinase kinase (MAPKKK), plays a negative role in the ethylene signaling pathway and *edr1* mutants show enhanced leaf senescence under ethylene treatment (Tang and Innes, 2002; Tang et al., 2005). A wheat (*Triticum aestivum*) ethylene receptor homolog (W-er1), with a histidine kinase domain, is induced during jasmonate and ABA triggered leaf senescence (Ma and Wang, 2003). The Arabidopsis SnRK2s (sucrose non-fermenting 1 related protein kinase 2), which have a positive function in the ABA signaling pathway, can phosphorylate ABA-responsive element binding factors (ABFs) and RAV1 (related to abi3/vp1 1) transcript factors to activate the expression of senescence associated genes in ABA-induced leaf senescence (Gao et al., 2016; Zhao et al., 2016). MPK6 (mitogen-activated protein kinase 6) has a regulatory role in both jasmonic acid- and salicylic acid- mediated leaf senescence (Yue et al., 2012; Chai et al., 2014; Zhang et al., 2016). The MKK4/5-MPK1/2 (mitogen-activated protein kinase kinase 4/5-MPK1/2) cascade regulates SA-induced leaf senescence through phosphorylation of NPR1 (non-expresser of PR genes 1) (Zhang et al., 2020). Mutants in BRI1 (Brassinosteroid insensitive 1), a component of the brassinosteroids receptor complex, show dark-green leaves and delayed senescence (He et al., 2007). Cytokinins are perceived by the histidine kinase receptors: AHK2, AHK3, and AHK4 (Arabidopsis His-kinase 2/3/4). Mutations in all three genes lead to shorter leaf longevity and loss of the ability to retain chlorophyll under cytokinin treatment in dark-induced leaf senescence (Riefler et al., 2006). AHK3 is the major cytokinin receptor involved in the control of leaf longevity by phosphorylation of ARR2 (a response regulator 2), an important transcription factor involved in the cytokinin signaling transduction pathway (Kim et al., 2006). Exogenous application of indole-acetic acid (IAA) negatively regulates leaf senescence (Kim et al., 2011), while Several SAURs (small auxin up-regulated RNA) are positive regulators of leaf senescence (Kant et al., 2009; Hou et al., 2013; Bemer et al., 2017). Therefore the detailed functions of auxin in leaf senescence remain controversial. Recent research showed that SAURs functions in accelerating the leaf senescence process via the activation of SARK-mediated leaf senescence signaling by suppressing SSPP (senescence suppressed protein phosphatase) (Wen et al., 2020).

Reactive oxygen species, comprised of singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO), are naturally generated as metabolic by-products in chloroplasts, mitochondria, peroxisomes and the apoplast of plants. ROS are highly toxic due to their reactive properties, which result in severe damage to cellular macromolecules, such as lipids, proteins, and nucleic acids. However, ROS are also known to play important roles in sensing and signal transduction in response to various biotic and abiotic stimuli and during developmental processes in plants (Møller et al., 2007; Swanson and Gilroy, 2010; Waszczak et al., 2018). OXI1 (oxidative signal-inducible 1), a serine/threonine protein kinase of the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family, is a downstream component of ROS signals and is activated by oxidative stress and wounding (Rentel et al., 2004). $^1O_2$ can lead to programmed cell death through the action of OXI1 at high light levels. Arabidopsis OXI1 over-expressing lines display hypersensitivity to high light and early senescence even in normal light conditions (Shumbe et al., 2016; Beaugelin et al., 2019). Most plant ABC1 atypical kinase (ABC1K, activity of bc1 complex kinase) proteins are located in either chloroplasts or mitochondria and are involved in the response to stresses (Lundquist et al., 2012). OsABC1-2, a rice ABC1K protein, encodes a chloroplast envelope-localized protein primarily present in green tissues. The null *osabc1-2* mutants have small plant size and pale-green leaves, and the *OsABC1-2* overexpressing lines show enhanced tolerance to prolonged dark-induced leaf senescence (Gao et al., 2012). The Arabidopsis ABC1K7 and ABC1K8 are involved in ROS homeostasis (Manara et al., 2015). *ABC1K7* and *ABC1K8* are upregulated by ABA, and the single *abc1k7* and *abk1k8* mutants and the double *abc1k7 abk1k8* mutants exhibit faster senescence rate than wild type plant under ABA treatment (Manara et al., 2016). However, the Arabidopsis plastoglobules-localized kinases ABC1K1 and ABC1K3 play roles in the regulation of high light stress induced leaf senescence, with a ROS-independent manner. The *abc1k1* and *abc1k3* mutants display rapid chlorosis in high light stress, and the double mutants show slow and irreversible senescence-like phenotype in moderate light caused by increased levels of jasmonate biosynthesis and pheophytinase activity, which accelerate chlorophyll degradation (Lundquist et al., 2013). In addition, the MAPK (mitogen-activated protein kinase) cascade is the classical signal transduction pathway in response to ROS (Jalmi and Sinha, 2015), in which MEKK1 and MPK6 are activated by ROS and reported to be involved in the aging process of plant (Nakagami et al., 2006; Miao et al., 2007; Zhou et al., 2009). Kinases related to ROS-regulated leaf senescence are summarized in **Figure 2** and **Supplementary Table 2**.

Ca^{2+} is a ubiquitous second messenger with an important signaling role in various stresses and developmental processes. Except for rapid and/or spatially restricted expanding cell, the concentration of resting cytosolic Ca^{2+} ($[Ca^{2+}]$) is kept approximately 100–200 nM, due to its potential toxicity at

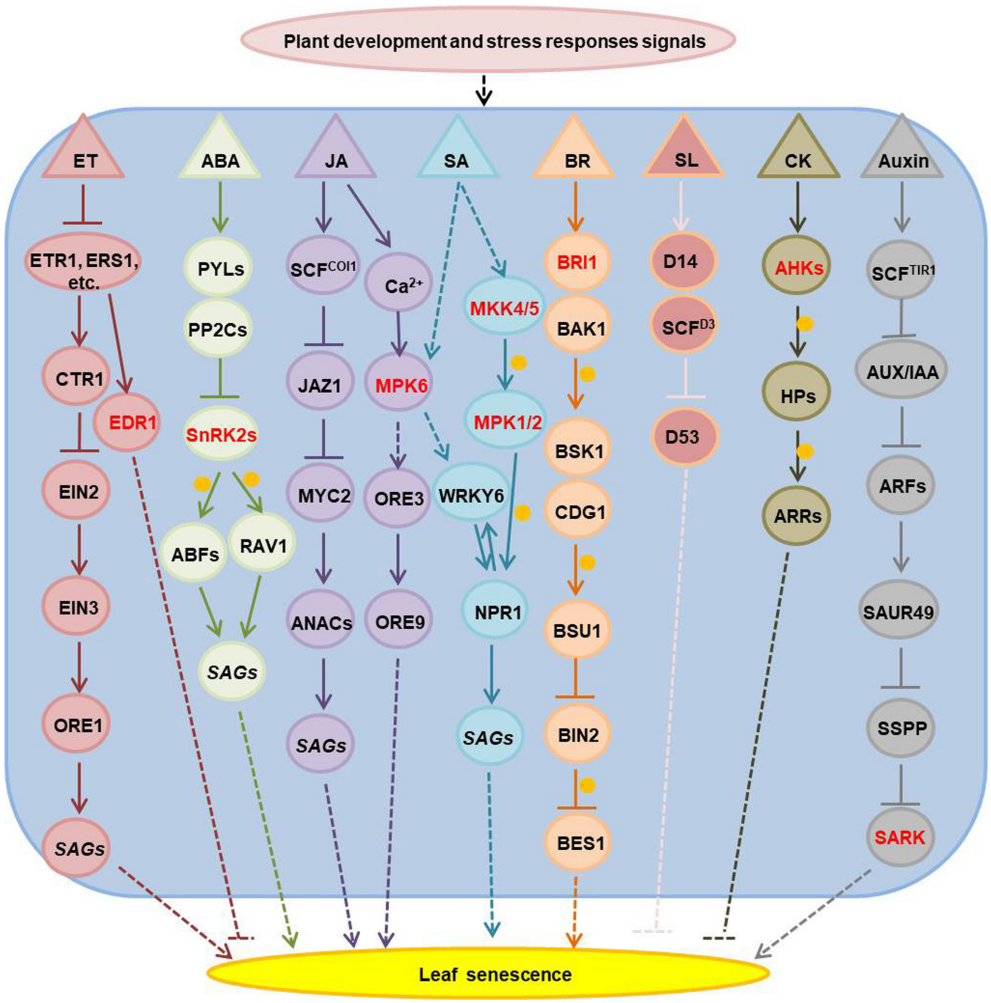

FIGURE 1 | The senescence-associated kinases functioned in hormones-regulated leaf senescence. The kinases are marked with red; arrows show that the process is promoted, and T-bars mean the process is inhibited; the solid lines indicate direct reported relationship, and the dotted lines indicate indirect reported relationship; the orange circles indicate phosphorylation. ET, ethylene; ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid; BR, brassinosteroids; SL, strigolactone; CK, cytokinins; ETR1, ethylene response 1, it is one of the five ET receptors; ERS1, ethylene response sensor 1, one of ET receptors; CTR1, constitutive triple response 1, homologous to the RAF family of serine/threonine protein kinases, a negative regulator in the ethylene signal transduction pathway, it interacts with the putative ethylene receptors ETR1 and ERS1; EIN2, ethylene insensitive 2, it acts downstream of CTR1 to regulate EIN3 positively; EIN3, ethylene-insensitive 3, a nuclear transcription factor that initiates downstream transcriptional cascades for ethylene responses; ORE1, ORESARA 1, it is a NAC-domain transcription factor and regulates senescence in leaves positively; *SAGs*, senescence associated genes; EDR1, enhanced disease resistance 1, a RAF family of serine/threonine protein kinases like CTR1, it has a negative role in ET signal pathway; PYLs, pyrabactin resistance 1-like family proteins, function as the ABA receptors; PP2Cs, the type 2C protein phosphatases, function as ABA co-receptors; SnRK2s, sucrose non-fermenting 1-related protein kinase 2 family proteins, they are activated by ABA and inhibited by PP2Cs; ABFs, ABA responsive element binding factor proteins, as leucine zipper transcription factors that bind to the ABA-responsive element (ABRE) motifs in the promoter region of ABA-inducible genes; RAV1, related to ABI3/VP1 1, an AP2/B3 domain transcription factor which is upregulated in ABA-induced leaf senescence; COI1, coronatine insensitive 1, JA receptor, it associates with AtCUL1, AtRbx1, and the Skp1-like proteins to assemble SCF^{COI1} ubiquitin-ligase complexes; JAZ1, jasmonate-zim-domain protein 1, it is degraded by SCF^{COI1} ubiquitin-ligase complexes under JA stimulus; MYC2, MYC-related transcriptional activator 2; ANACs, NAC domain-contained transcription factors; MPK6, mitogen-activated protein kinase (MAPK) 6; ORE3, ORESARA 3 or named as EIN2; ORE9, ORESARA 9, as a member of the F-box leucine-rich repeat family proteins, it is a proposed regulator of leaf senescence; MKK4/5, MAPK kinase 4/5; MPK1/2 (MAPK1/2), mitogen-activated protein kinase 1/2; BRI1, BR insensitive 1, encodes a plasma membrane localized leucine-rich repeat receptor kinase, as BR receptor; BAK1, BRI1-associated receptor kinase, as the BR co-receptor with BRI1, it is a leucine-rich receptor serine/threonine protein kinase; BSK1, BR-signaling kinase 1; CDG1, constitutive differential growth 1, is a receptor-like cytoplasmic kinase, belongs to RLCKVII subfamily; BSU1, BRI1 suppressor 1, encodes a serine-threonine protein phosphatase; BIN2, brassinosteroid-insensitive 2, a member of the ATSK (shaggy-like kinase) family; BES1, BRI1-EMS-suppressor 1, a key transcription factor involved in BR signaling, coordinates plant growth and stress responses; D14, is a receptor in the SL signaling pathway; SCF^{D3}, as a member of the F-box leucine-rich repeat family of proteins, they are involved in SCF-dependent protein ubiquitination; D53, interacts with D14 in an SL-dependent manner, and it is shown to be degraded through the 26S proteasome pathway in a manner that requires the function of the F-box protein D3; AHKs, Arabidopsis histidine kinases, CK receptors, including AHK2, AHK3, and AHK4; HPs, histidine-containing phosphotransfer proteins; ARRs, Arabidopsis response regulators, including type-A and type-B ARR; TIR1, transport inhibitor response 1, encodes an auxin receptor, it contains leucine-rich repeats and an F-box and forms SCF (Skp-Cullin-F-box) complexes with ASK1 and CUL1; AUX/IAA, repressors of auxin-responsive transcription; ARFs, auxin-response factors; SAUR, small auxin upregulated RNA; SSPP, senescence suppressed protein phosphatase; SARK, senescence-associated receptor-like kinase.

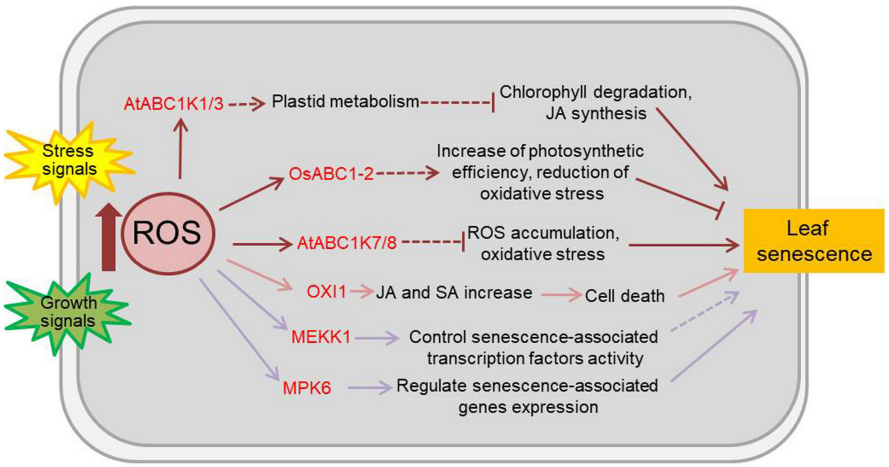

FIGURE 2 | Effects of ROS on plant leaf senescence. Numerous abiotic and biotic stresses like salinity, heat, cold, nutrients, heavy metals, insects, pathogens, etc., resulting to the accumulation of ROS and the change of kinase activity. As the main and important second messenger in plants, ROS participate in a number of physiological responses and development process including leaf senescence. The kinases functioned in ROS-regulated leaf senescence are marked in red. Arrows show that the process is promoted, and T-bars mean the process is inhibited; solid lines indicate the direct reported relationship, and dotted lines indicate indirect reported relationship. OXI1, oxidative signal-inducible 1; ABC1 atypical kinase, activity of bc1 complex kinase; MEKK1, MAPK/ERK kinase kinase 1; MPK6, mitogen-activated protein kinase 6; JA, jasmonic acid; SA, salicylic acid.

higher levels, but organelles and the extracellular space can reach millimolar Ca^{2+} concentrations. Consequently, a steep [Ca^{2+}] concentration gradient is established between the cytosol and the different Ca^{2+} stores. One of the most intriguing aspects of Ca^{2+} signaling is the complex spatio-temporal patterns of Ca^{2+} influx, including concentration, amplitude, duration and oscillation induced in cells by various stimuli. Free Ca^{2+} is sensed and decoded by several types of Ca^{2+}-binding proteins with EF-hand motifs. Calmodulin (CaM), a highly conserved eukaryotic protein with four EF-hand domains, is involved in the regulation of multiple interacting proteins (e.g., transcription factors). Calcineurin B-like (CBL) proteins are regulatory proteins without enzymatic activity *per se*, but they interact with specific CBL-interacting protein kinases (CIPKs), which are activated upon CBL binding (Steinhorst and Kudla, 2013). Calcium-dependent protein kinases (CDPKs or CPKs) have an N-terminal variable domain, a protein kinase domain, an auto-inhibitory junction domain, and a C-terminal calmodulin-like domain (Atif et al., 2019). Many calcium-related kinases are involved in the regulation of leaf senescence (**Figure 3** and **Supplementary Table 3**). AtCIPK14 has an indirect negative effect in leaf senescence by phosphorylating the transcription factor WHY1 (WHIRLY1). Once phosphorylated by AtCIPK14, the accumulation of WHY1 increased in nucleus, promoting its binding to the promoter of *WRKY53* and thus decreasing the expression of several *SAGs* (Ren et al., 2017). ESL4 (early senescent leaf 4), a rice CDPK, is involved in nitrogen metabolism and leaf senescence, with *esl4* mutants showing premature leaf senescence when grown under low-nitrogen conditions (Xing et al., 2018). The rice OsCPK12 plays a role in leaf senescence by regulating ROS levels and photosynthetic rate (Wang B. et al., 2019). Overexpression of the maize *ZmCPK11* in Arabidopsis, improves salt tolerance by preventing salt-induced chlorophyll degradation and damage to photosystem II (Borkiewicz et al., 2020). The *Brassica napus* transcription factor BnaWSR1 binds to the promoter of *ICS1* (*isochorismate synthase 1*), *RbohD* (*respiratory burst oxidase homolog protein D*), and *SAG14* (*senescence associated gene 14*) to regulate their expression, resulting in the accumulation of SA and ROS during the leaf senescence process. BnaCPK5/6 (*B. napus* Calcium-dependent protein kinase 5/6) interacts with and phosphorylates BnaWSR1 (*B. napus* WRKY regulating SA and ROS 1) to enhance its transcriptional activity, thus BnaCPK5/6 is involved in cell death and leaf senescence (Cui et al., 2020). BnaCPK2 and BnaCPK6L (*B. napus* Calcium-dependent protein kinase 2; *B. napus* Calcium-dependent protein kinase 6) interact with and phosphorylate BnaRBOHD (*B. napus* Respiratory burst oxidase homolog D) both to enhance BnaRBOHD activity and generate more ROS in cell which would accelerate cell death and leaf senescence (Wang et al., 2018; Pan et al., 2019). A CDPK-related kinase (CRK) AtCRK3, is involved in regulation of leaf senescence in Arabidopsis by phosphorylating the cytosolic glutamine synthetase AtGLN1;1/AtGSR1 (*A. thaliana* glutamine synthase clone R 1), important for nitrogen remobilization and reutilization during leaf senescence (Li R. et al., 2006).

Reactive oxygen species, Ca^{2+} and phytohormone are involved in the regulation of almost all growth stages and stress responses in plants. The phytohormones function always integrated with ROS, Ca^{2+}, and it has been studied extensively (Shabala et al., 2016; Choi et al., 2017; Demidchik and Shabala, 2018; Demidchik et al., 2018). The association of ROS and Ca^{2+} has been newly defined, although the relationship between them remains elusive. It was found that not only ROS has been reported to regulate Ca^{2+} channels activity (Demidchik, 2015; Choi et al., 2017), but Ca^{2+} could also induce ROS generation by activating NADPH-oxidase simultaneously

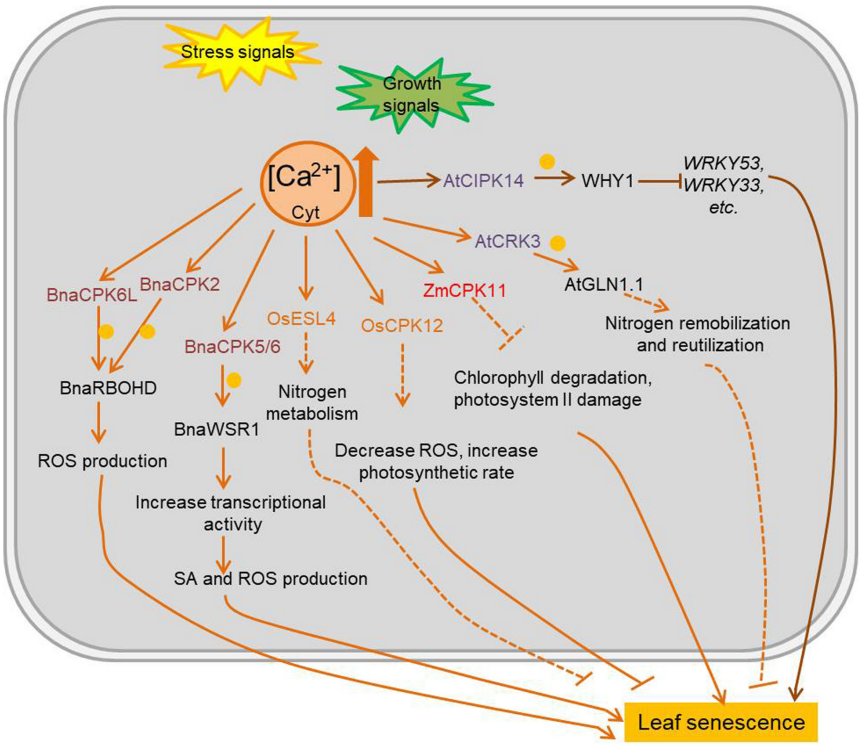

FIGURE 3 | Ca^{2+} mediated plant leaf senescence in different conditions. Intracellular Ca^{2+} change rapidly during plant development and stress response, and it always acts as second messenger to activate Ca^{2+} sensing proteins. The kinases which are regulated by levels of Ca^{2+} and function in leaf senescence are marked in colors. Arrows show that the process is promoted, and T-bars mean the process is inhibited; blue circles indicate phosphorylation; solid lines indicate the direct reported relationship, and dotted lines indicate indirect reported relationship. CPKs/CDPKs, calcium-dependent protein kinases; CIPKs, CBL-interacting protein kinases; CBL, calcineurin B-like proteins; OsESL4, *O. sativa* early senescent leaf 4; AtCRK3, *Arabidopsis thaliana* CDPK-related kinase 3; BnaWSR1, *B. napus* WRKY regulating SA and ROS 1; AtGSR1, *Arabidopsis thaliana* glutamine synthase clone R 1; WHY1, WHIRLY1; JA, jasmonic acid; SA, salicylic acid.

(Kobayashi et al., 2007; Yamauchi et al., 2017). The relationship among ROS, Ca^{2+} and phytohormone were complex and still obscure, all of them were found to function during leaf senescence process, although more and more studies were reported in recent years, therefore, the coordination among them in the regulation of leaf senescence was more complex.

KINASES INVOLVED IN PLANT IMMUNITY AND LEAF SENESCENCE

Among the evolutionarily conserved pathways, the mitogen-activated protein kinase (MAPK) cascade signaling pathways have been identified as important regulators of development and environmental responses in plants, especially plant immunity (Zhang et al., 2018). A typical MAPK cascade consists of at least three sequentially acting serine/threonine kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and finally, a MAP kinase (MAPK), with each phosphorylating, and hence activating the next kinase in the cascade. MAPK modules are activated in response to extracellular and/or intracellular signals and play key roles in the transduction of environmental and developmental signals through phosphorylation of downstream signaling targets, ultimately triggering major changes in gene expression and adaptive physiological responses. MAPK targets include kinases, enzymes, cytoskeletal proteins and transcription factors (Xu and Zhang, 2015; Krysan and Colcombet, 2018). There are about 80 MAPKKKs, 10 MAPKKs, and 20 MAPKs in Arabidopsis, some of which being involved in several signaling networks having an integrative function in the plants response to their environment (Chardin et al., 2017; Jagodzik et al., 2018). A variety of transcriptome analysis revealed a large number of MAPKs kinases with altered expression patterns during leaf senescence (Buchanan-Wollaston et al., 2003; Guo et al., 2004; Breeze et al., 2011; Guo and Gan, 2012). An Arabidopsis MAPKKK, MEKK1 (MAP kinase or ERK kinase kinase 1) affects leaf senescence by binding with an important senescence transcription factor WRKY53 (Miao et al., 2007), while the MEKK1-MKK1/2-MPK4 cascade negatively regulates innate immune responses (Gao et al., 2008; Kong et al., 2012); Another Arabidopsis MAPKKK kinase, EDR1 (enhanced disease resistance 1), plays a negative role in powdery mildew resistance and ethylene induced leaf senescence (Tang and Innes, 2002); the rice MAPKKK, SLES (spotted leaf sheath) is involved in disease resistance and leaf senescence by regulating the dynamic balance of ROS (Lee et al., 2018). The MKK9-MPK6 cascade in Arabidopsis positively regulates leaf senescence (Zhou et al., 2009), and also have a role in

melatonin-mediated innate immunity (Lee and Back, 2016). The Arabidopsis MKK4/5-MPK1/2 cascade mediates salicylic acid induced leaf senescence (Zhang et al., 2020), while MEKK1-MKK4/5-MPK6 is activated by bacterial and fungal pathogens (Asai et al., 2002). MPK6 participates in jasmonate and salicylic acid induced plant senescence (Yue et al., 2012; Chai et al., 2014; Zhang et al., 2016), and has a role in plant defense (Pitzschke et al., 2009; Thulasi Devendrakumar et al., 2018). In addition to the mentioned kinases, there are other MAPK cascade components involved in the regulation of senescence, although it is not clear whether they have roles in the plant immune response. For instance, Arabidopsis MAPKKK18 positively regulates aging and ABA induced senescence (Matsuoka et al., 2015). Arabidopsis MAPK1/6/7 phosphorylate TTM1 to regulate its function and turnover of TTM1 during ABA triggered leaf senescence (Karia et al., 2021). Rice MAPKKK1 (SPL3, spotted leaf 3) positively regulates leaf senescence via the ABA signaling pathway (Wang S. et al., 2015). In maize, the ZmMEK1-ZmSIMK1 (*Zea mays* MAP kinase or ERK kinase-*Zea mays* salt-induced mitogen-activated protein kinase 1) cascade is involved in salicylic acid mediated leaf senescence (Li et al., 2016), while the ZmMKK10-ZmMPK3/7 cascade plays a role in ethylene-dependent cell death (Chang et al., 2017), and the ZmMPK5 kinase activity is enhanced in senescent leaves (Berberich et al., 1999). Although there are many MAPKs involved in the leaf senescence process, it is not clear how these MAPK cascades perceive and are activated by senescence signals.

In addition to MAPKs, other types of kinases are jointly involved in plants defense and leaf senescence. The Rice lesion mimic mutant *lmm24*, identified as a receptor-like cytoplasmic kinase 109, is involved in the regulation of cell death and plant defense (Zhang et al., 2019). BAK1 (BRI1-associated receptor kinase), initially identified as a brassinosteroid co-receptor together with BRI1, has a much wider role as co-receptor of multiple pattern recognition receptors (PRR) involved in the regulation of cell death and plant immunity (He et al., 2007; Heese et al., 2007; Schwessinger et al., 2011; Wu et al., 2020). Increased expression of wheat stripe rust resistance protein WKS1 (wheat kinase-start 1) in transgenic wheat accelerate leaf senescence, due to the phosphorylation of the thylakoid-associated ascorbate peroxidase tAPX reducing its ability to detoxify peroxides (Gou et al., 2015). Kinases with known roles in plant defense as well as leaf senescence are listed in **Figure 4** and **Supplementary Table 4**.

ENERGY AND METABOLISM ASSOCIATED KINASES INVOLVED IN LEAF SENESCENCE

As sessile organisms, plants have to endure many environmental changes, which may deplete their energy stores. To survive such challenges, plants possess many energy sensors to maintain energy homeostasis. Among the energy sensors, there are a number of kinases with important roles in the regulation of plant growth, development, and stress tolerance (Doorn, 2008), including SnRK1 (sucrose non-fermenting 1 related protein kinase 1), TOR (the target of rapamycin), ATGs (autophagy-related proteins), etc. Energy and metabolism-related kinases involve in the regulation of leaf senescence are listed in **Figure 5** and **Supplementary Table 5**.

SnRK1 is one of the evolutionarily conserved energy sensor proteins in plants. Upon activation by sugar starvation or energy depletion in cells, SnRK1 phosphorylates downstream key enzymes and induce extensive changes in gene expression patterns (Broeckx et al., 2016). There are two *SnRK1* genes in Arabidopsis, *SnRK1.1* (also known as *KIN10* or *AKIN10*) and *SnRK1.2* (also known as *KIN11* or *AKIN11*). Transgenic Arabidopsis plants overexpressing *SnRK1.1* display delayed flowering time and leaf senescence (Baena-González et al., 2007; Cho et al., 2012), however, overexpression of *SnRK1.2* leads to flower early (Williams et al., 2014). The Arabidopsis transcription factor bZIP63 plays a positive role in dark-induced senescence, and its function is repressed by SnRK1.1-mediated phosphorylation during starvation-induced senescence (Mair et al., 2014). The Arabidopsis SnRK1.1 plays a negative role in the ethylene-induced senescence process by phosphorylating the important transcription factor in ethylene signaling EIN3 (ethylene-insensitive 3) leading to its destabilization (Kim et al., 2017). The maize *SnRK1* gene family is composed of three functional members, *ZmSnRK1.1*, *ZmSnRK1.2*, and *ZmSnRK1.3*. Overexpression of all *ZmSnRK1s* in Arabidopsis results in delayed leaf senescence (Wang J. et al., 2019). The negative role of plant SnRK1 proteins in the regulation of leaf senescence maybe a strategy for plants to maintain cell viability and avoid sudden death under unfavorable conditions.

Target of rapamycin (TOR), an atypical Ser/Thr protein kinase that belongs to the phosphoinositide 3-kinase-related kinase family, is a central coordinator of nutrient, energy, hormone and stress signaling networks in plants (Ren et al., 2011). TOR forms kinase complexes with regulatory proteins, and these TOR interacting partners play a role in recruiting and regulating diverse TOR substrates. The TOR kinase complex comprises TOR, RAPTOR (regulatory-associated protein of TOR), and LST8 (lethal with SEC13 protein 8) in plants. There is one *TOR gene*, two *Raptor* (*RaptorA*, *RaptorB*) genes, and two *LST8* (*LST8-1, LST8-2*) genes in Arabidopsis (Xiong and Sheen, 2014). The members of the TOR complex are vital for integrating internal and external cues to regulate plant growth and development. TOR null mutants are embryo lethal; inducible RNA interference lines are small leaf size, shorter root length, early senescence, and low seed production, while *TOR*-overexpressing plants display the opposite phenotypes (Deprost et al., 2007; Ren et al., 2011). *lst8-1* mutants show modest dwarf growth and accelerated senescence (Moreau et al., 2012).

Plant autophagy is a highly conserved catabolic process in which cells encapsulate and deliver cytoplasmic components into the vacuole for degradation and recycling of essential nutrients (Li and Vierstra, 2012; Liu and Bassham, 2012). Autophagy is primarily induced by natural senescence and a variety of unfavorable environmental factors, which will lead to nutrient limitation and accelerated nutrient recycle, e.g., nutrient deprivation, high salt, drought, hypoxia, oxidative stress, pathogen infection (Marshall and Vierstra, 2018a). There

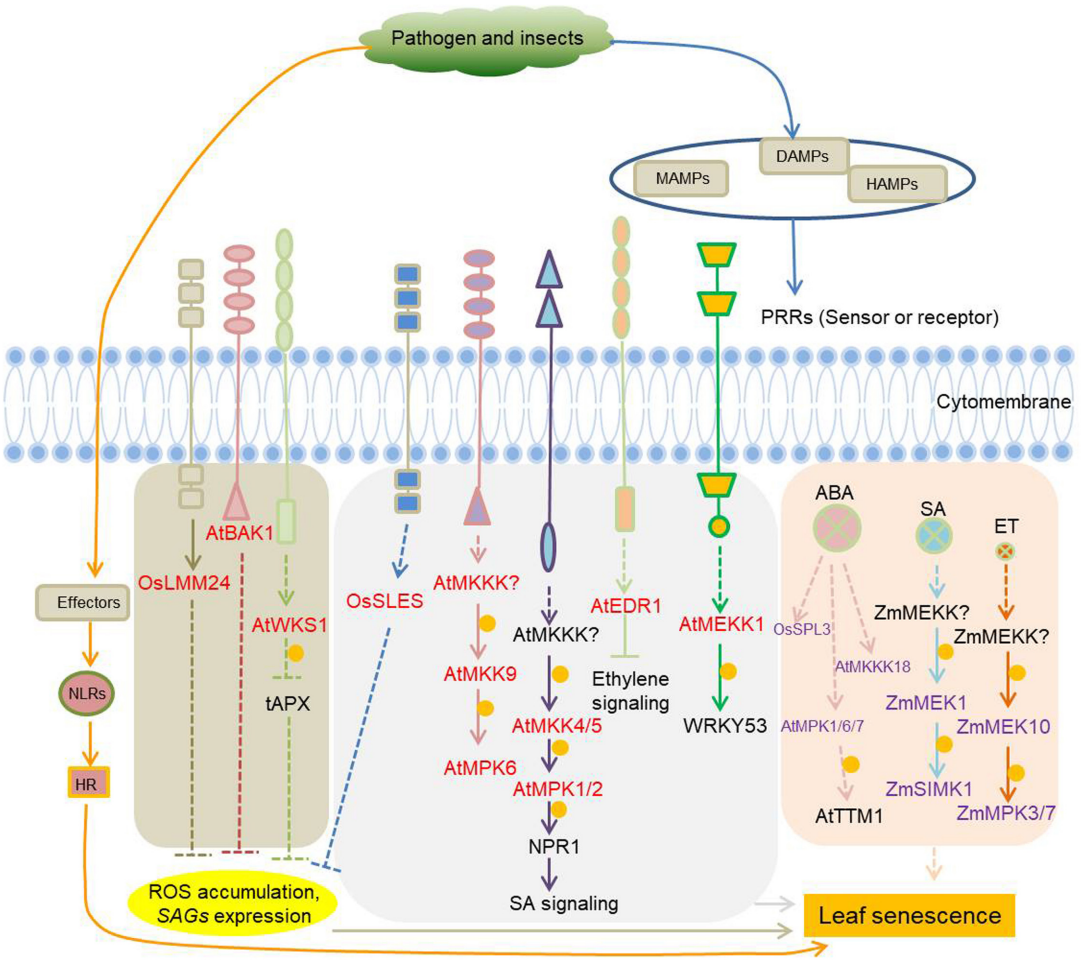

FIGURE 4 | Protein kinases involved in the regulation of plant immunity-related leaf senescence. Pathogens and pests induce microbial-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) or herbivore-associated molecular patterns (HAMPs) that can be recognized by specific plant receptors (pattern recognition receptors, PRRs) to initiate cell-surface immunity. PRRs are receptor-like kinases (RLKs) or receptor-like proteins (RLPs) in plants. RLKs are composed of an extracellular ligand binding domain, a transmembrane region, and an intracellular kinase domain. RLPs have a similar structural organization to RLKs, but lack the kinase domain. Pathogens/pests can also deliver elicitors/effectors to inside of cells, and these elicitors/effectors can be sensed by intracellular immune receptors (NLRs) to initiate intracellular immunity, which will lead to hypersensitive response (HR), a form of programmed cell death (PCD). Cell-surface immunity and intracellular immunity activate downstream short-term and long-term defense responses, respectively. The kinases involved in plant immunity-mediated leaf senescence are marked in red. The kinases marked in purple are leaf senescence regulars, whether they function in response to immunity is unknown. Arrows show that the process is promoted, and the T-bars mean the process is inhibited. Yellow circles indicate phosphorylation. OsLMM24, *O. sativa* lesion mimic mutant 24; BAK1, BRI1-associated receptor kinase; WKS1, wheat kinase-start 1; tAPX, thylakoid-associated ascorbate peroxidase; OsSLES, *O. sativa* spotted leaf sheath; MKKKs or MEKKs, MAPK kinase kinases; MKKs or MEKs, MAPK kinases; NPR1, non-expresser of PR genes 1; EDR1, enhanced disease resistance 1; OsSPL3, *O. sativa* spotted leaf 3.

are a large number of autophagy-related proteins (ATGs) in plants with essential roles in regulation of autophagy (Soto-Burgos et al., 2018; Yoshimoto and Ohsumi, 2018). Plant ATG complexes are grouped into four functional categories: (1) proteins that initiate autophagy, including the ATG1 kinase core complex, containing four subunits: ATG1/ATG13/ATG17-ATG29-ATG31/ATG11; (2) proteins that mediate emergence of phagophores, including the ATG9 kinase complex, containing three subunits: ATG9/ATG2/ATG18; (3) factors that remodel autophagic membranes, including the class III phosphatidylinositol-3-kinase (PI3K) complex, containing the VPS34 (Vacuolar protein sorting 34), VPS15, ATG6 and ATG14 four subunits; (4) two ubiquitin-like conjugation complexes, ATG5-ATG12 and ATG8-PE (phosphatidylethanolamine), which decorate phagophores and autophagosomes (Marshall and Vierstra, 2018b; Marshall et al., 2019). Arabidopsis ATG mutants display premature leaf senescence and shortened life cycle even under normal growth conditions, hypersensitivity to nutrient deficiency, decreased tolerance to biotic and abiotic stresses, activated innate immunity, and an altered cellular metabolism (Doelling et al., 2002; Xiong et al., 2007; Liu et al., 2009; Hayward and Dinesh-Kumar, 2011; Guiboileau et al., 2012; Li et al., 2014; Avin-Wittenberg et al., 2015; Qi et al., 2020). Interestingly, both the TOR kinase and SnRK1 are involved in

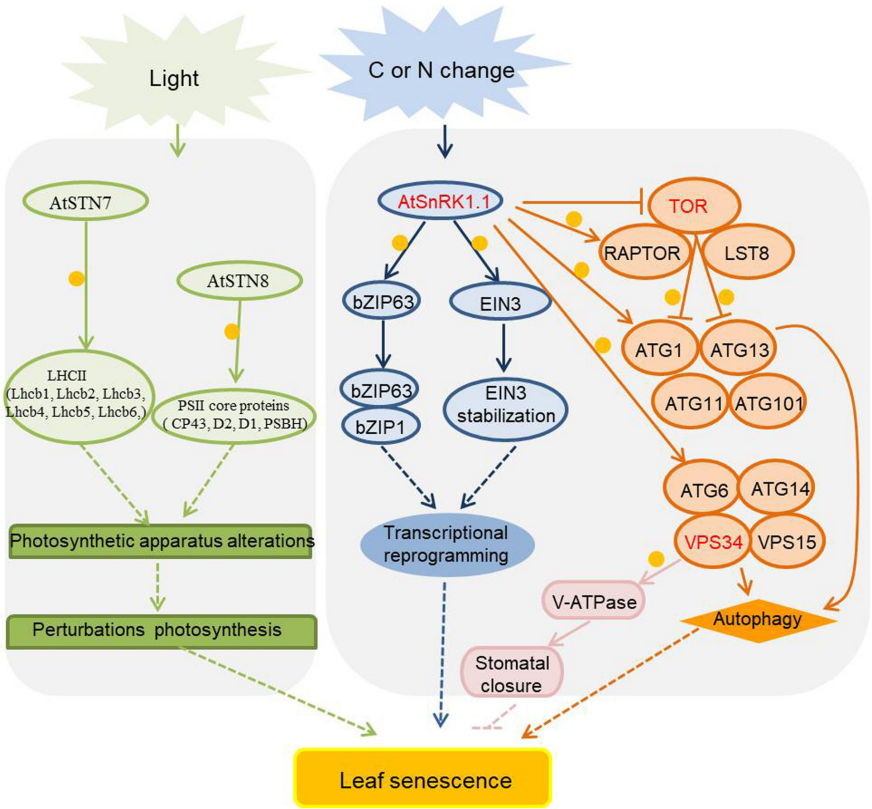

FIGURE 5 | Energy and metabolism related kinases in the regulation of leaf senescence. As metabolite, carbon (C) and nitrogen (N) assimilates are transported from source organs to sink organs, and the nutrient balance of carbon and nitrogen plays an important role in signaling transduction during leaf senescence. The activities of protein kinases are affected by sugar and nitrogen signals and participate in the aging process of plants, and they are marked in red, orange circles indicate phosphorylation. Arrows show that the process is promoted, and T-bars mean the process is inhibited. The activity of SnRK1 is induced by starvation, and it phosphorylates some transcription factors to regulate *SAGs* expression or to induce the autophagy process by phosphorylating ATG1 and ATG6. SnRK1 also phosphorylates RAPTOR to inhibit the TOR's activity. TOR negatively regulates autophagy process by phosphorylating ATG1 and ATG13 to inhibit the initial formation of autophagosomes. The PI3K protein VPS34 phosphorylates V-ATPase to activate stomatal acidification and promote stomatal closure during JA-induced leaf senescence. STN7 and STN8 maintain the balance of photosystems by phosphorylating PSII core and LHCII protein, when the phosphorylation status changed drastically, the balance would be disturbed and the leaves would senescence. STN7/8, state transitions 7/8; PSII, Photosystem II; LHCII, light-harvesting complex II; SnRK1, sucrose non-fermenting 1 related protein kinase 1; EIN3, ethylene-insensitive 3; TOR, target of rapamycin; RAPTOR, regulatory-associated protein of mTOR; LST8, lethal with SEC13 protein 8; PI3K, phosphoinositide 3-kinase; ATGs, autophagy-related proteins; VPS34, vacuolar protein sorting 34; VPS15, vacuolar protein sorting 15; V-ATPase, vacuolar H^+-ATPase.

autophagy by phosphorylation of ATGs. Under nutrient-rich conditions, TOR phosphorylates the ATG13 and ATG1 subunits to prevent autophagy. Meanwhile, in nutrient starvations conditions SnRK1.1 phosphorylates ATG1 and ATG6 to induce autophagy (Chen et al., 2017; Pu et al., 2017; Soto-Burgos and Bassham, 2017; Huang et al., 2019). Moreover, PI3K interacts with V-ATPase (vacuolar H^+-ATPase) to activate stomatal acidification, which leads to stomatal closure and delayed leaf senescence, and also alleviates leaf senescence under jasmonate treatment (Liu et al., 2016a,b).

STN7 and STN8 (state transitions 7/8) are important chloroplast kinases that can phosphorylate different photosynthesis-associated thylakoid proteins to adapt to environmental changes (Bellafiore et al., 2005; Bonardi et al., 2005). The primary function of STN7 is the phosphorylation of LHCII (light-harvesting complex II) triggering its migration to PSI (photosystem I) to initiate a state transition. STN8 phosphorylates PSII (photosystem II) core proteins to modulate thylakoid ultrastructure and facilitates the repair of damaged PSII. STN7 and STN8 help to maintain optimal activity of the photosynthetic apparatus and have a crucial role in short-term acclimation and long-term responses (Vainonen et al., 2005; Puthiyaveetil et al., 2012; Poudyal et al., 2020). Interestingly, both loss-of-function and overexpression of *STN7* and *STN8* result in early onset of senescence, suggesting that any perturbations of these two genes-regulated acclimation processes will induce early senescence in plants (Wang J. et al., 2015).

CONCLUSION AND PERSPECTIVES

It is an important approach to reveal molecular mechanism of leaf senescence by investigating genetic mutants with altered leaf senescence process. Many kinase-associated

mutants and/or transgenic plants were detected earlier/delayed leaf senescence phenotype and these materials played roles to find new components and their regulatory networks involved in the leaf senescence process (Li Z. et al., 2020). A large number of *SAGs* have been found by differential expression techniques in different plants (Guo et al., 2004; Buchanan-Wollaston et al., 2005; Breeze et al., 2011; Guo and Gan, 2012), and some SAGs were protein kinases, which play roles in signal transduction during leaf senescence. The researchers have found lots of SAPs (senescence associated proteins) through proteomics approaches and combined the information of metabolites change by metabolomics during leaf senescence, however, no protein kinase was detected as SAPs because of their low abundance in nature (Hebeler et al., 2008; Watanabe et al., 2013; Balazadeh et al., 2014; Moschen et al., 2016; Wei et al., 2016). Phosphoproteomic data identified many phosphorylation motifs, and it showed us potential kinase-substrate or kinase phosphorylation site during leaf senescence, moreover, the information of co-expression kinases and external co-localization or co-interaction were also supplied by phosphoproteomic data (Mergner et al., 2020), further work to find new protein kinase in the regulation of leaf senescence or study the function of protein kinase in leaf senescence by taking advantage of this data. Despite many kinases as the senescence regulators have been found involved in leaf aging, the substrates of most leaf aging-related kinases are still unknown, which is vital to discover the entire signaling cascades or pathway during leaf senescence. The proteome and metabolite profiling analyses are effective approaches to expand and verify transcriptomics-induced molecular responses. Integration of multi-omics data including genomic, transcriptomic, proteomic, and metabolomic of leaf senescence wound provide a possible pathway to find the potential kinase-substrate combination during leaf senescence and reveal their molecular function. Therefore, considering the importance and complexity of signaling pathway in leaf senescence, and the vital roles of protein kinases in signal transduction, the in-depth study on leaf senescence using integrated omics approaches would help to unravel the key issues in leaf senescence, such as how and when plant initiate, execute and finish leaf senescence process, what is the initiate signal of leaf senescence, what are the differences between natural leaf senescence and stress-induced leaf senescence. Finally, studies on screening and functional analysis of senescence associated kinases are directly linked with growth and breeding, it is the cornerstone for improving crop production.

Phosphorylation by protein kinases has a strong effect on the conformation, activity, stability, and localization of target proteins. Leaf senescence is an integral part of plant development, and it is affected by internal and external factors. The different kinases involved in the regulation of leaf senescence play vital roles in the perception of senescence-associated information and transmission of the signal to downstream factors. Although a large number of kinases have been implicated in the regulation of plant leaf senescence, further work is needed to build the connections between the different components of the senescence process, and novel signaling components and pathways will continue to be discovered. Elucidation of the senescence mechanisms associated with environmental fitness and reproduction could be used to enhance stress tolerance and improve crop yield.

AUTHOR CONTRIBUTIONS

FY, KL, and C-PS wrote the manuscript. YM, WL, YL, and JB commented on the first draft and critically reviewed the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.864215/full#supplementary-material

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REVIEW
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New Advances in the Regulation of Leaf Senescence by Classical and Peptide Hormones

Peixin Huang[1], *Zhonghai Li*[1]* *and Hongwei Guo*[1,2]*

[1] Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, National Engineering Research Center for Tree Breeding and Ecological Restoration, College of Biological Sciences and Technology, Beijing Forestry University, Beijing, China, [2] Key Laboratory of Molecular Design for Plant Cell Factory of Guangdong Higher Education Institutes, Department of Biology, Southern University of Science and Technology, Shenzhen, China

Leaf senescence is the last stage of leaf development, manifested by leaf yellowing due to the loss of chlorophyll, along with the degradation of macromolecules and facilitates nutrient translocation from the sink to the source tissues, which is essential for the plants' fitness. Leaf senescence is controlled by a sophisticated genetic network that has been revealed through the study of the molecular mechanisms of hundreds of senescence-associated genes (SAGs), which are involved in multiple layers of regulation. Leaf senescence is primarily regulated by plant age, but also influenced by a variety of factors, including phytohormones and environmental stimuli. Phytohormones, as important signaling molecules in plant, contribute to the onset and progression of leaf senescence. Recently, peptide hormones have been reported to be involved in the regulation of leaf senescence, enriching the significance of signaling molecules in controlling leaf senescence. This review summarizes recent advances in the regulation of leaf senescence by classical and peptide hormones, aiming to better understand the coordinated network of different pathways during leaf senescence.

Keywords: leaf senescence, plant hormones, peptide, senescence-associated gene, regulatory network

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Balakumaran Chandrasekar, Birla Institute of Technology and Science, India

***Correspondence:**
Zhonghai Li
lizhonghai@bjfu.edu.cn
Hongwei Guo
guohw@sustech.edu.cn

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INTRODUCTION

Leaf senescence occurs as the final step of leaf development, preceding the ultimate cell death or completion of life cycle (Pennell and Lamb, 1997; Lim et al., 2007). Leaves are the important organ that can store energy in form of carbohydrate molecules converted from light energy through photosynthesis. As leaves age, photosynthetic efficiency declines and chloroplasts degrade, accompanied by the degradation of macromolecules such as lipids, proteins, and nucleic acids (Gan and Amasino, 1997; Lim et al., 2007). Leaf senescence is crucial for plant development and fitness because the nutrients released from senescent leaves are reallocated to other developing young organs for better reproductive growth in annual plants, or to be stored in phloem tissues for successful growth of next season in perennial plants (Cooke and Weih, 2005; Lim et al., 2007). Premature or delayed leaf senescence evidently reduces the yield and quality of crop plants such as rice and wheat (Buchanan-Wollaston et al., 2005; Srivalli and Khanna-Chopra, 2009; Breeze et al., 2011; Su et al., 2017; Piao et al., 2019). Studying regulatory mechanisms of leaf senescence will provide instructive hints for precise improvement of agronomic yield and quality.

The onset of leaf senescence depends mainly on the developmental age, as demonstrated by the fact that only plants older than 24 days induced significant yellowing on cotyledons after ethylene

treatment, while plants younger than 17 days did not (Jing et al., 2002). It is also influenced by various endogenous and environmental factors. Out of them, plant hormones play pivotal roles in the regulation of leaf senescence. Basically, cytokinins (CKs), auxin, and gibberellins (GAs) delay leaf senescence, whereas ethylene, salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), brassinosteroids (BRs), and strigolactones (SLs) accelerate senescence (Gan and Amasino, 1995; Li et al., 1996, 2013; Morris et al., 2000; Kim et al., 2011; Jibran et al., 2013; Yamada and Umehara, 2015; Zhu et al., 2015; Mao et al., 2017; Guo et al., 2021). Hormones not only directly regulate leaf senescence process, but also link environmental signals into the modulation of leaf senescence (Yang et al., 2011; Zhang et al., 2019). Briefly, plant hormones regulate leaf senescence through the following pathways: (i) affect leaf growth and development and alter the state of leaves that can be used to induce senescence; (ii) influence the progression and features of senescence via genetic transduction; (iii) integrate the environmental signals into developmental processes. Recently, peptide hormones were found to participate in the regulation of leaf senescence (Aghdam et al., 2021a; Zhang et al., 2022b), and studies on mechanism of senescence regulated by peptide hormones enrich the knowledge to understand regulation of leaf senescence. In this review, we provide an overview and highlight new advances in the regulation of leaf senescence by classical and peptide hormones. Major components of hormones biosynthesis and signaling involving in leaf senescence are presented (**Table 1**).

PLANT HORMONES THAT DELAY LEAF SENESCENCE

Cytokinins

Cytokinins (CKs) are N^6-substituted adenine derivatives that regulate diverse aspects of plant growth and development processes, including shoot meristems, vascular development, root growth, nodulation, as well as leaf initiation and leaf senescence (Argueso et al., 2010; Perilli et al., 2010; Wu et al., 2021). CK works as a negative regulator of senescence, which was supported by the evidences that exogenous application CK retards senescence and endogenous CKs decrease during senescence (Singh et al., 1992; Gan and Amasino, 1996). Expressions of CK synthesis-associated genes decrease and a CK oxidase involving CK degradation is up-regulated when the senescence occurs, which is consistent with the gradually decrease of CKs content along with senescence (Buchanan-Wollaston et al., 2005). The expression of isopentenyl transferase (*IPT*) driven by a senescence-specific promoter *SAG12* obviously delays leaf senescence process (Gan and Amasino, 1995). The autoregulatory *proSAG12:IPT* system has been widely utilized in numerous important crop plants that exhibit retarded leaf senescence, indicative of the negative regulation of leaf senescence by CK. Therefore, the researchers manipulated the leaf senescence process by regulating the CK content through molecular genetic pathways. Overexpression of *FPS1S* (Farnesyl diphosphate synthase) leads to the declined endogenous zeatin-type CK with a concomitant senescence-like phenotype in *Arabidopsis* (Masferrer et al., 2002). SlymiRNA208 suppresses the post-transcriptional expression of *SlIPT2* and *SlIPT4* in tomato, resulting in the premature leaf senescence in the *SlymiRNA208*-overexpressing plants by reducing the endogenous concentration of CKs (Zhang et al., 2020b). APT1 (Adenine phosphoribosyl transferase 1), which catalyzes the CK conversion from free bases to nucleotides, acts as a positive regulator of leaf senescence. Loss of APT1 activity causes a delayed leaf senescence due to the excess accumulation of CKs (Zhang et al., 2013b).

The CK signaling pathway initiates with the binding of CKs to histidine kinase receptors, known as AHK2, AHK3, and CRE1/WOL/AHK4, then involves a phosphotransfer cascade, and ultimately triggers transcription of CK-responsive genes in the nucleus (Argueso et al., 2010). Several components of CK signal transduction are found to function in leaf senescence (**Table 1**). Gain-of-function of AHK3 leads to the extended leaf longevity, conversely *ahk3* loss-of-function mutant exhibits early leaf senescence during dark-induced senescence (Kim et al., 2006). AHK3 mediates the specific phosphorylation of downstream type-B response regulator ARR2 that plays a crucial role in CK-mediated leaf longevity. Consistently, the plants overexpressing *ARR2* show delayed leaf senescence during dark-induced and age-dependent senescence, but not overexpression of $ARR2^{D80N}$, in which the phosphotransfer to ARR2 is abolished (Hwang and Sheen, 2001; Kim et al., 2006). Interestingly, the inhibition of ARR2 degradation through a substitution of Lys90 with Gly also delays leaf senescence (Kim et al., 2012). Although AHK3 plays a major role in CK-dependent chlorophyll retention in the detached leaves, AHK2 and AHK4 also contribute to CKs-mediated leaf longevity (Riefler et al., 2006). Cytokinin response factors (CRFs) are transcriptionally induced by CK and act downstream of AHK3 to regulate leaf senescence. Plants with overexpressing *CRF6* possess a higher chlorophyll retention than wild type without exogenous CK (Zwack et al., 2013), unraveling CRF6 as a negative regulator during dark-induced leaf senescence. In contrast, simultaneous silencing of CRF1/3/5/6 delays leaf senescence and overexpression of CRF1/3/5 accelerates senescence, accompanied by an induction of *SAG12* and reduction of *CAB2* (Raines et al., 2016). The different roles in regulating leaf senescence imply that CRFs are involved in regulating the leaf senescence process through different pathways downstream of CK.

CKs participate in the regulation of sink/source relations during leaf senescence, which partially depends upon the activity of cell-wall invertase (CWINV) (Godt and Roitsch, 1997; Balibrea Lara et al., 2004). CWINV and hexose transporters are effective enzymes in mediating the phloem unloading process of sucrose. Expression of CWINV under the control of *SAG12* promoter (*proSAG12:CWINV*) delays leaf senescence (Godt and Roitsch, 1997; Balibrea Lara et al., 2004). Further, a link between CK and CWINV underlying leaf senescence is substantiated by the evidence that expression of an invertase inhibitor driven by a CK-inducible promoter does not show delayed senescence in the presence of CKs (Balibrea Lara et al., 2004; Jin et al., 2009). Taken together, CWINV is an essential component that mediates CK-conferred leaf longevity. Besides, CKs influence leaf senescence via interaction with other hormones. For

TABLE 1 | List of the key components in hormone pathway involved in leaf senescence.

Gene	Hormone	Effect	Species	Reference
IPT	CK	Delay	*Arabidopsis thaliana*	Gan and Amasino, 1995
FPS1S	CK	Promote	*Arabidopsis thaliana*	Masferrer et al., 2002
SlymiRNA208	CK	Promote	*Solanum lycopersicon*	Zhang et al., 2020b
APT1	CK	Promote	*Arabidopsis thaliana*	Zhang et al., 2013b
AHK3	CK	Delay	*Arabidopsis thaliana*	Kim et al., 2006
AHK2, AHK4	CK	Delay	*Arabidopsis thaliana*	Riefler et al., 2006
ARR2	CK	Delay	*Arabidopsis thaliana*	Kim et al., 2006
CRF6	CK	Delay	*Arabidopsis thaliana*	Zwack et al., 2013
YUC6	Auxin	Delay	*Arabidopsis thaliana*	Kim et al., 2011
ZmGH3.8	Auxin	Delay	*Zea mays*	Feng et al., 2021
IAA17	Auxin	Promote	*Arabidopsis thaliana*	Shi et al., 2015
ARF2	Auxin	Promote	*Arabidopsis thaliana*	Lim et al., 2010
ARF1, ARF7, ARF19	Auxin	Promote	*Arabidopsis thaliana*	Ellis et al., 2005
ANT	Auxin	Delay	*Arabidopsis thaliana*	Feng et al., 2016
SAUR36	Auxin	Promote	*Arabidopsis thaliana*	Hou et al., 2013
SAUR39	Auxin	Promote	*Oryza sativa*	Kant et al., 2009
SAUR49	Auxin	Promote	*Arabidopsis thaliana*	Wen et al., 2020
BrGA20ox3	GA	Delay	*Brassica rapa*	Xiao et al., 2019
GAI, RGA, RGL1, RGL2	GA	Delay	*Arabidopsis thaliana*	Chen et al., 2014
ScGAI	GA	Delay	*Saccharum spp.*	Fang et al., 2021
ACS	Ethylene	Promote	*Arabidopsis thaliana*	Tsuchisaka et al., 2009
ETR1, ERS1	Ethylene	Delay	*Arabidopsis thaliana*	Qu et al., 2007
EIN2	Ethylene	Promote	*Arabidopsis thaliana*	Oh et al., 1997
EIN3, EIL1	Ethylene	Promote	*Arabidopsis thaliana*	Chao et al., 1997
miRNA164	Ethylene	Delay	*Arabidopsis thaliana*	Li et al., 2013
ORE1	Ethylene	Promote	*Arabidopsis thaliana*	Kim et al., 2009
ORS1, AtNAP, ANAC019/047/055	Ethylene	Promote	*Arabidopsis thaliana*	Kim et al., 2014
ZmNAC126	Ethylene	Promote	*Zea mays*	Yang et al., 2020
ERF4, ERF8	Ethylene	Promote	*Arabidopsis thaliana*	Koyama et al., 2013
SID2	SA	Promote	*Arabidopsis thaliana*	Abreu and Munne-Bosch, 2009
PAD4	SA	Promote	*Arabidopsis thaliana*	Morris et al., 2000
WHY1	SA	Delay	*Arabidopsis thaliana*	Lin et al., 2020
S3H	SA	Delay	*Arabidopsis thaliana*	Zhang et al., 2013a
S5H/DMR6	SA	Delay	*Arabidopsis thaliana*	Zhang et al., 2017b
NPR1	SA	Promote	*Arabidopsis thaliana*	Morris et al., 2000
PVA31	SA	Promote	*Arabidopsis thaliana*	Ichikawa et al., 2015
LOX1, LOX2, LOX3, LOX4	JA	Promote	*Arabidopsis thaliana*	He et al., 2002
KAT2	JA	Promote	*Arabidopsis thaliana*	Castillo and Leon, 2008
TaWRKY13-A	JA	Promote	*Triticum aestivum*	Qiao et al., 2021
TaWRKY42-B	JA	Promote	*Triticum aestivum*	Zhao et al., 2020
miR139	JA	Delay	*Arabidopsis thaliana*	Schommer et al., 2008
TCP2, TCP4, TCP10	JA	Promote	*Arabidopsis thaliana*	Schommer et al., 2008
COI1	JA	Promote	*Arabidopsis thaliana*	Castillo and Leon, 2008
COS1	JA	Delay	*Arabidopsis thaliana*	Xiao et al., 2004
JAZ7	JA	Delay	*Arabidopsis thaliana*	Yu et al., 2016
MYC2, MYC3, MYC4	JA	Promote	*Arabidopsis thaliana*	Zhu et al., 2015; Yu et al., 2016
Dof2.1	JA	Promote	*Arabidopsis thaliana*	Zhuo et al., 2020
OsERF101	JA	Promote	*Oryza sativa*	Lim et al., 2020
MdBT2, MdJAZ2	JA	Delay	*Malus pumila Mill.*	An et al., 2021a
ESR/ESP	JA	Delay	*Arabidopsis thaliana*	Miao and Zentgraf, 2007

(Continued)

TABLE 1 | Continued

Gene	Hormone	Effect	Species	Reference
PSF	ABA	Delay	*Oryza sativa*	Wang et al., 2016
ES3(t)	ABA	Delay	*Oryza sativa*	Su et al., 2017
PvCCCH69	ABA	Delay	*Panicum virgatum*	Xie et al., 2021
CsHB5	ABA	Promote	*Citrus reticulata Blanco.*	Zhang et al., 2021d
OsNAC2	ABA	Promote	*Oryza sativa*	Mao et al., 2017
CDF4	ABA	Promote	*Arabidopsis thaliana*	Xu et al., 2020
OsMYB102	ABA	Delay	*Oryza sativa*	Piao et al., 2019
AAO3	ABA	Promote	*Arabidopsis thaliana*	Yang et al., 2014
SAG113	ABA	Promote	*Arabidopsis thaliana*	Zhang et al., 2012
ABA2	ABA	Delay	*Arabidopsis thaliana*	Song et al., 2016
PYL8	ABA	Promote	*Arabidopsis thaliana*	Lee et al., 2015
PYL9	ABA	Promote	*Arabidopsis thaliana*	Zhao et al., 2016
ABIG1	ABA	Promote	*Arabidopsis thaliana*	Liu et al., 2016
ABF2, ABF3, ABF4	ABA	Promote	*Arabidopsis thaliana*	Gao et al., 2016
OsNAP	ABA	Promote	*Oryza sativa*	Liang et al., 2014
ONAC054	ABA	Promote	*Oryza sativa*	Sakuraba et al., 2020
ABI5	ABA	Promote	*Arabidopsis thaliana*	Su et al., 2016
DET2	BR	Promote	*Arabidopsis thaliana*	Li et al., 1996
UGT73C6	BR	Delay	*Arabidopsis thaliana*	Husar et al., 2011
CYP105A1	BR	Delay	*Arabidopsis thaliana*	Dasgupta et al., 2011
DRL1/BAT1	BR	Delay	*Agrostis stolonifera L.*	Han et al., 2017
BRI1	BR	Promote	*Arabidopsis thaliana*	Li and Chory, 1997
BES1	BR	Promote	*Arabidopsis thaliana*	Yin et al., 2002
AIF2	BR	Delay	*Arabidopsis thaliana*	Kim et al., 2020
CCD7	SL	Promote	*Lotus japonicus*	Liu et al., 2013
CCD8	SL	Promote	*Petunia hybrida*	Snowden et al., 2005
ORE9/MAX2	SL	Promote	*Arabidopsis thaliana*	Woo et al., 2001
CLE14	Peptide	Delay	*Arabidopsis thaliana*	Zhang et al., 2022b
CLE42	Peptide	Delay	*Arabidopsis thaliana*	Zhang et al., 2022a
PSKR1	Peptide	Delay	*Arabidopsis thaliana*	Matsubayashi et al., 2006
TPST	Peptide	Delay	*Arabidopsis thaliana*	Komori et al., 2009

example, CKs reduce ABA content through suppressing the transcription of ABA synthesis-related genes but elevating the expression of ABA degradation genes, which leads to the retarded leaf senescence (Zhang et al., 2021c). Given the regulatory mechanism of CKs in sink/source relations is largely unknown, more investigations needs to be done to unravel the role of CKs in the regulation of leaf senescence, especially regarding sink/source relations, which will be useful for application in molecular breeding.

Auxin

Auxin not only functions in cell growth in response to environmental stimuli, but also fulfills an important role in leaf initiation, morphogenesis, as well as senescence (Vanneste and Friml, 2009). Indole-3-acetic acid (IAA), a major natural auxin, transiently inhibits the expression level of *SAG12* (Noh and Amasino, 1999), indicating that exogenous auxin represses leaf senescence process. Surprisingly, the endogenous IAA levels detected in senescent leaves were 2-fold higher than in fully expanded young leaves (Quirino et al., 1999; van der Graaff et al., 2006). Although this increase may be due to non-uniform regulation between cells in the senescent and non-senescent parts, the significance of this increase during leaf senescence is not clear. A recent study on the effect of IAA on gene expression during leaf senescence reported that IAA treatment accelerated the progression of senescence-related changes, and furthermore, it revealed that the earlier treatment time, i.e. 27 DAS (a few days after sowing), resulted in the most significant acceleration of late leaf senescence compared to 35 DAS (Goren-Saglam et al., 2020). Further microarray analysis of gene expression of IAA treatment at different time points showed that the effect of IAA on leaf senescence was not only time-dependent but also interacted with ethylene and JA pathways. Therefore, discussion of the function of IAA in leaf senescence requires consideration of the balance of endogenous hormone networks.

Activating or mutating components of the auxin pathway in planta helps us to better understand the function of IAA in leaf senescence. YUCCAs (YUCs) encoding flavin monooxygenases

catalyze a restrictive step in auxin biosynthesis, namely the conversion from indole-3-pyruvic acid (IPA) to auxin (Kim et al., 2007). Activation of YUC6 in *Arabidopsis* increases free IAA levels, reduces expression of SAGs and exhibits a delayed senescence phenotype (Kim et al., 2011). The thiol-reductase activity of YUC6 also mediates reactive oxygen species (ROS) content and auxin availability to influence leaf senescence (Cha et al., 2016). Auxin signal is perceived by its receptor protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN SIGNALING F BOX PROTEINs (AFBs), leading to the ubiquitin-mediated degradation of AUX/IAA proteins, which are repressors of auxin response factors (ARFs) (Vanneste and Friml, 2009). Then activation of auxin response genes is accompanied by the release of ARFs. The plant overexpressing signaling component AtIAA17 displays early leaf senescence with lower chlorophyll content in rosette leaves, conversely *iaa17* mutant shows a delayed senescence phenotype (Shi et al., 2015). ARF2, a transcriptional repressor of auxin signaling, is induced in senescing leaves. The *arf2* mutant displays the delayed senescence symptoms of rosette leaves in natural and dark conditions (Lim et al., 2010). AINTEGUMENTA (ANT), a member of the AP2/ERF TF family, is demonstrated to act downstream of ARF2 to extend leaf longevity (Feng et al., 2016). Moreover, mutations in *ARF7* and *ARF19*, two transcriptional activators, enhance *arf2* phenotype (Ellis et al., 2005), which indicates that auxin is involved in the regulation of leaf senescence by controlling gene expression in manifold ways. The early auxin-responsive genes, including *SMALL AUXIN-UP RNA* (*SAURs*) genes such as *SAUR36*, *SAUR39*, and *SAUR49*, are involved in leaf senescence (Kant et al., 2009; Hou et al., 2013; Wen et al., 2020). The soybean (*Glycine max*) SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE (GmSARK) and its ortholog in *Arabidopsis* AtSARK act as positive regulators of leaf senescence through a widespread mechanism relating to auxin, ethylene, and cytokinin (Xu et al., 2011). SAUR49 promotes leaf senescence via activation of SARK-mediated signaling by repressing SENESCENCE SUPPRESSED PROTEIN PHOSPHATASE (SSPP) (Wen et al., 2020). Therefore, SAURs-SARK regulation mode may be significant for integrating the senescence signals and hormone signaling in plants. Recently, modification of autophagy and auxin signals via manipulating expression of *ZmATG18b* and *ZmGH3.8* gene alters the time of maize leaf senescence (Feng et al., 2021). Besides, IAA29 is involved in the PIF4 and PIF5-mediated regulation of heat stress-induced leaf senescence (Li et al., 2021b). Taken together, these studies suggest that auxin may interact with other hormones and environmental signals to coordinate plant growth and the onset of leaf senescence at the right time.

Gibberellins

Gibberellins (GAs) are a class of tetracyclic diterpenoid, some of which are bioactive in regulating many aspects of plant growth and development, such as stem elongation, leaf expansion, seed dormancy and germination, plant flowering, and response to abiotic and biotic stresses (Gao and Chu, 2020). The content of endogenous GAs declines as leaves age, and exogenous application of GA_3 retards the degradation of chlorophyll (Aharoni, 1978; Li et al., 2010), indicating that GAs repress the progression of senescence. The GA 2-oxidase 2 (GA2OX2) gene, which causes GA inactivation, is up-regulated 18-fold during leaf senescence (van der Graaff et al., 2006), suggesting that the decrease in active GA may be a cause of leaf senescence. TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) TF BrTCP21 directly binds the promoter of GA biosynthetic gene *BrGA20ox3*, activates its transcription, and delays leaf senescence (Xiao et al., 2019). The transcript of *BrTCP21* decreases along with leaf senescence, while GA_3 treatment keeps *BrTCP21* expression in a higher level, which suggests that the positive feedback loop of GA-BrTCP21-GA plays an important role in leaf senescence. In *Arabidopsis*, GA signal is received by the receptor GID1 and then transduced to release the repression of TFs by negative regulator DELLA proteins, including GAINSENSITIVE (GAI), REPRESSOR OF GA1-3 (RGA), RGA-LIKE1 (RGL1), RGA-LIKE2 (RGL2), and RGA-LIKE3 (RGL3) (Olszewski et al., 2002; Hedden and Sponsel, 2015). The natural leaf senescence occurs earlier when four DELLA proteins (*gai-t6 rga-t2 rgl1-1 rgl2-1*) are knocked-out (Chen et al., 2014), which suggests what appears to be a contradiction with GA inhibition of leaf senescence. Since GA is an important regulator of plant flowering and the *gai-t6 rga-t2 rgl1-1 rgl2-1* mutant has an early flowering phenotype, it cannot be simply assumed that GA promotes senescence. Therefore, the functions of DELLA and GA cannot be equated in the regulation of leaf senescence. In supporting this hypothesis, DELLA proteins delay leaf senescence by interacting with and suppressing the functions of WRKY45, WRKY6, WRKY75, and NAP, positive regulators of leaf senescence (Chen et al., 2017; Zhang et al., 2018a, 2021b; Lei et al., 2020). Similarly, ScGAI delays age-trigged senescence by interacting with and then repressing the function of ScNAC23 in sugarcane (Fang et al., 2021). Additionally, GAs might indirectly regulate leaf senescence via crosstalk with other hormones. The decline of GA level is usually accompanied by an increase of ABA content, and exogenous GA_3 treatment could inhibit the surge of ABA during leaf senescence (Yu et al., 2009), implying an antagonistic effect of GA and ABA in regulation of leaf senescence.

PLANT HORMONES THAT ACCELERATE LEAF SENESCENCE

Ethylene

Ethylene is a well-known gas phytohormone that acts as an endogenous facilitator of plant aging, including the senescence processes of leaf and petal, as well as fruit ripening. Exogenous application of ethylene or increase in endogenous ethylene content promotes leaf senescence, while inhibitors of ethylene biosynthesis retard senescence (Abeles et al., 1988; Grbić and Bleecker, 1995; Wang et al., 2001). Ethylene does not directly determine the onset of leaf senescence, since ethylene only accelerates the progression of leaf senescence when leaves reach a defined age (Jing et al., 2002, 2005). Transcription analysis reveals that a number of genes involving ethylene biosynthesis and signaling components are regulated in senescent leaves (van der Graaff et al., 2006). 1-aminocyclopropane-1-carboxylate (ACC) synthases (ACS) are biosynthetic enzymes to produce the key precursor of ethylene, and the *acs octuple* mutant exhibits a

prominently delayed senescence (Tsuchisaka et al., 2009). The role of ethylene in the control of leaf senescence is also explained by the function of signaling elements, as evidenced by the *etr1-1*, *ein2*, and *ein3 eil1* mutants with extended leaf longevity (Grbić and Bleecker, 1995; Chao et al., 1997; Oh et al., 1997), and *etr1 ers1* with earlier senescence (Qu et al., 2007), which is consistent with the positive effect of ethylene in regulating leaf senescence.

ETHYLENE-INSENSITIVE2 (EIN2) is a central positive regulator of ethylene signaling and mediates most of the ethylene response. Expression of *ORE1/NAC092*, one target of miR164, is up-regulated by EIN2 during gradually leaf aging, while *miR164* expression is suppressed by EIN2 (Kim et al., 2009). The trifurcate feed-forward pathway involving ORE1, miR164 and EIN2 finally results in increased expression of *ORE1* that promotes leaf senescence. ETHYLENE-INSENSITIVE3 (EIN3) is a master transcription factor in ethylene signaling, and acts downstream of EIN2 to regulate ethylene response. EIN3 represses *miR164* transcription via directly binding the promoter of *miR164*, leading to increased transcript levels of *ORE1* (Li et al., 2013). The linear pathway involving EIN2-EIN3-miR164-ORE1 sheds light on accelerated leaf senescence by ethylene regulation. However, EIN2 does not fully depend on ORE1 in regulating senescence, since EIN2 still contributes to senescence-associated cell death in the absence of ORE1. More senescence-associated NAC transcription factors are found to act as the downstream components of EIN2 governing leaf senescence, including ANAC019, AtNAP, ANAC047, ANAC055, and ORS1 (Kim et al., 2014). WRKY71 functions as a positive regulator of leaf senescence by communicating with ethylene signal in *Arabidopsis*. WRKY71 is an ethylene-inducible gene and influences leaf senescence by directly regulating EIN2 and ORE1 (Yu et al., 2021b). EIN3 and ORE1 induce the chlorophyll degradation through directly activating chlorophyll catabolic genes (Qiu et al., 2015). ZmNAC126 is transactivated by ZmEIN3 and regulates chlorophyll degradation of ethylene-induced senescence in maize (Yang et al., 2020). Therefore, an intricate transcription network involving EIN2 and EIN3 plays significant roles in ethylene-triggered leaf senescence. AP2/ERF transcription factors such as AtERF4 and AtERF8, activated by ethylene, also involve in modulating the onset of leaf senescence (Koyama et al., 2013; Koyama, 2014). Additionally, ethylene interacts with other hormones to influence leaf senescence (Kim et al., 2015; Iqbal et al., 2017). For example, the detached leaves of *ein2* and *ein3 eil1* mutants are insensitive to MeJA-induced leaf senescence compared to that in wild type (Li et al., 2013), indicating that JA-induced leaf senescence is dependent upon ethylene signal. Ethylene is thought to be a downstream signal that promotes the progression of leaf senescence in an age-dependent manner. However, the mechanisms involved are not fully understood. Figuring out the relationship between ethylene and age information and how age information is encoded will really help to understand the nature of leaf senescence.

Salicylic Acid

Salicylic acid (SA) is a phenolic hormone involved in plant development, abiotic and biotic stress adaption. It is critical for defense against plant pathogens, especially as a component of systemic acquired resistance (Malamy et al., 1990; Metraux et al., 1990). SA content gradually increases in the senescing leaves of multiple species (Zhang et al., 2017b), and SA deficiency mediated by transgenic *NahG* line and defect in *SID2*, an isochorismate synthase of SA biosynthesis, delays leaf senescence in *Arabidopsis* (Abreu and Munne-Bosch, 2009), depicting a connection between SA and leaf senescence. The intact SA signaling pathway contributes to control the expression of senescence-enhancing genes whose increased transcripts are disrupted by mutations in NPR1 or PAD4 (Morris et al., 2000). PHYTOALEXIN DEFICIENT4 (PAD4) promotes SA accumulation, especially in response to pathogen infection (Makandar et al., 2015). PAD4-dependent SA pathway has a central role in *saul1* mutant-mediated initiation of leaf senescence to induce visible symptoms of senescence, and activation of senescence in the aphid-infested leaves (Pegadaraju et al., 2005; Vogelmann et al., 2012). Leaf senescence induced by SA is associated with SA-dependent cell death, since *pad4* mutant exhibits a delayed yellowing and reduced necrosis at the final stage of senescence (Morris et al., 2000). The retrograde signaling protein WHIRLY1 (WHY1) alters its organelle isoforms in nucleus or chloroplasts, and perturbs SA homeostasis via regulating expression of SA biosynthesis genes *SID2* and *PAL1* (Lin et al., 2020). SA 3-hydroxylase (S3H) and S5H are involved in the SA catabolism by catalyzing SA conversion into 2,3- and 2,5-dihydroxybenzoic acid, and the defect in S3H and S5H over-accumulates active SA content, leading to an early senescence (Zhang et al., 2013a, 2017b). Thus, the active SA content, accompanied by regulation of SA homeostasis plays an essential role in promoting leaf senescence. A number of WRKY TFs are unraveled to influence leaf senescence via modulating SA pathway by different modes. WRKY75, WRKY28, WRKY55, WRKY40, WRKY46, WRKY51, WRKY60, and WRKY63 activate the expression of *SID2* by binding to its promoter, thus augment the accumulation of SA to accelerate leaf senescence (Guo et al., 2017; Zhang et al., 2017a; Tian et al., 2020; Wang et al., 2020). WRKY TFs are also able to affect senescence through indirectly regulating the biosynthesis and signaling of SA, for example, WRKY28 mediates SA biosynthesis in response to light signals (Tian et al., 2020). WRKY46 also interacts with NPR1, the core component of SA signal transduction, to improve *WRKY6* expression to mediate probenazole/SA-elicited leaf senescence (Zhang et al., 2021a).

SA pathway integrates many signals or physiological processes to change states of leaf senescence. Recently, two groups simultaneously reveal that SA coordinates with ethylene to accelerate leaf senescence, achieved by interaction of NPR1 and EIN3 to promote expression of SAGs synergistically (Wang et al., 2021; Yu et al., 2021a). In addition, SA signaling is involved in leaf senescence induced by autophagy, PVA31-mediated membrane trafficking, membrane phospholipid metabolism, and ROS (Yoshimoto et al., 2009; Xiao et al., 2010; Ichikawa et al., 2015; Guo et al., 2017). In summary, SA plays an important role in the onset and development of leaf senescence and is coordinated by multiple factors. Since SA mediates both immunity and aging, SA

is the best link to explore the relationships between immunity and senescence.

Jasmonic Acid

Jasmonic acid (JA) is a class of oxylipin phytohormones derived from polyunsaturated fatty acids, preferentially α-linolenic acid (Li et al., 2021a). JA regulates myriad aspects of plant growth and development, as well as stress responses. JA accumulates in senescing leaves and positively regulates leaf senescence (He et al., 2002). JA biosynthesis-associated genes are differentially regulated during leaf senescence, including *LOX*, *AOS*, *AOC*, and thiolase (He et al., 2002). *LOX1*, *LOX3*, and *LOX4* are obviously up-regulated with the progression of leaf senescence, while *LOX2* is down-regulated (He et al., 2002). This difference implies different roles for LOXs in regulating senescence, such as the exclusive role of LOX2 in stress-induced leaf senescence (Seltmann et al., 2010). *KAT2* (*3-ketoacyl-CoA thiolase 2*), a gene encoding the JA-biosynthetic β-oxidation enzyme, is strongly activated in natural and dark-induced senescing leaves, while reduction of *KAT2* expression leads to significantly delayed senescence (Castillo and Leon, 2008). Several factors involve in regulation of leaf senescence through modulating JA biosynthesis. TaWRKY13-A and TaWRKY42-B facilitate the onset and progression of leaf senescence by promoting the expression of *LOX* genes, which consequently induces accumulation of JA content in wheat (Zhao et al., 2020; Qiao et al., 2021). miR139 indirectly controls JA biosynthesis via changing TCPs activity, thus overexpression of *miR139* delays leaf senescence (Schommer et al., 2008).

Components of JA signaling pathway take part in the regulation of leaf senescence. JA perception is achieved by receptors complex comprised of COI1 (CORONATINE INSENSITIVE1) and JAZ family proteins, subsequentially leading to the degradation of JAZ proteins via 26S-proteosome (Li et al., 2021a). JA-induced premature senescence is blocked in JA insensitive mutant *coi1* (He et al., 2002; Castillo and Leon, 2008), suggesting the intact JA pathway is necessary for senescence activation. Xiao et al. (2004) screened for the suppressors of *coi1* and isolated the *cos1* (*coi1 suppressor1*) mutant. Defect in *COS1* gene, which encodes lumazine synthase for riboflavin pathway, severely reduces the higher chlorophyll content in the *coi1* mutant compared with wild type (Xiao et al., 2004). The constant yellowing phenotype of *cos1 coi1* leaves points out a novel function of riboflavin pathway in regulating leaf senescence. As a negative regulator of JA signaling, JAZ7 is induced by darkness, thus disturbs the functions of downstream MYC2/MYC3/MYC4 TFs to suppress dark-induced leaf senescence (Yu et al., 2016). Meanwhile, the *jaz7* mutant exhibits more severe leaf yellowing and chlorophyll degradation (Yu et al., 2016). MYC2/MYC3/MYC4 are the master TFs of JA signaling pathway mediating JA-induced leaf senescence. JA-modulated leaf senescence is demonstrated to associate with regulation of ROS and chlorophyll degradation. For example, MYC2 represses the expression of *CATALASE 2* (*CAT2*) gene in JA-treated leaves, and the subsequent H_2O_2 accumulation leads to advanced leaf senescence (Zhang et al., 2020a). CAT2 mutation correctly rescuing delayed leaf senescence of *myc2* mutant further proves the important roles of ROS in JA-induced senescence. Moreover, MYC2/MYC3/MYC4 promote expression of chlorophyll catabolic genes, such as *PAO*, *NYC1*, and *NYE1*, by directly binding to their promoters, respectively, thus causing chlorophyll degradation, so that *myc2 myc3 myc4* exhibits a severe stay-green phenotype (Zhu et al., 2015). Additionally, MYCs proteins are not the only TFs participating in JA-regulated leaf senescence. ANAC019/055/072 exerts synergistic effects and bHLH subgroup IIId factors act antagonistically with MYCs to regulate JA-related leaf senescence (Qi et al., 2015; Zhu et al., 2015). Interestingly, the MYC2-Dof2.1-MYC2 feedforward transcriptional loop positively regulates dark-induced and age-dependent leaf senescence (Zhuo et al., 2020). In addition, the key components of JA signaling are also targeted by other factors to regulate leaf senescence process. For example, OsERF101 elevates the expression of *OsMYC2* and *OsCOI1a* to promote JA-mediated leaf senescence in rice (Lim et al., 2020). Apple MdBT2, a scaffold protein having ubiquitination activity, accelerates MdMYC2 degradation and stabilizes MdJAZ2 protein through direct interactions, thereby antagonistically regulates JA-activated leaf senescence (An et al., 2021a). Crosstalk between circadian clock and JA pathway finely cooperates many processes of plant growth and development. The Evening Complex (EC) in circadian oscillator negatively regulates JA-mediated leaf senescence via repressing the expression of *MYC2* (Zhang et al., 2018b, 2019). Furthermore, JA integrates with other endogenous phytohormones to affect leaf senescence. WRKY57 acts as a suppressor of JA-induced leaf senescence. Auxin antagonizes JA-induced leaf senescence process via up-regulating expression of WRKY57 (Jiang et al., 2014). Additionally, JAZ4/8 and IAA29, repressors of the JA and auxin signaling pathways respectively, competitively interact with WRKY57 (Jiang et al., 2014). Therefore, WRKY57 functions as an important integrator of JA and auxin pathways in leaf senescence modulation. JA-inducible ESR/ESP inhibits the functions of WRKY53, a positive regulator of leaf senescence. ESR/ESP and WRKY53 mediate leaf senescence on the basis of JA and SA homeostasis and the consequent regulation of these two genes antagonistically (Miao and Zentgraf, 2007). Autophagy up-regulated by low concentration SA alleviates JA-induced leaf senescence (Yin et al., 2020), further indicating a compact antagonistic interaction of SA and JA in leaf senescence. In summary, JA acts as an important integrative signal that communicates with other plant hormones to regulate leaf senescence and adjust the response to biotic and abiotic factors.

Abscisic Acid

Abscisic acid (ABA) is a kind of phytohormone constituted of sesquiterpenoid. ABA regulates a myriad of plant development processes, such as seed dormancy and germination, stomatal closure, shoot and root growth, leaf senescence, as well as abiotic responses (Chen et al., 2020; Sano and Marion-Poll, 2021). ABA induces premature leaf senescence, and endogenous ABA content is an important regulatory factor affecting leaf senescence. Phenotype of rice *psf* (*premature senescence of flag leaves*) mutant that exhibits premature senescence lesion in senescent leaves results from high level of ABA accumulation,

concomitantly with low rate of D1 protein synthesis and aggravated PSII photodamage during leaf senescence (Wang et al., 2016). The level of ABA content increases in the *early senescence 3* (*es3*) mutant, leading to the upregulation of SAGs (Su et al., 2017). Zinc finger protein PvCCCH69 suppresses age- and dark-induced leaf senescence via antagonizing ABA pathway (Xie et al., 2021). Several other factors modulate leaf senescence with regulation of ABA biosynthesis and metabolism. CsHB5, OsNAC2, and CDF4 elevate the expression of ABA biosynthetic genes, increase ABA content, and promote leaf senescence (Mao et al., 2017; Xu et al., 2020; Zhang et al., 2021d). OsMYB102 and OsNAC2 are involved in ABA metabolism via regulating expression of ABA catabolic enzymes (Mao et al., 2017; Piao et al., 2019). OsMYB102 inhibits ABA accumulation by inducing ABA catabolic gene *OsCYP707A6*, thereby delaying age- or dark-induced leaf senescence (Piao et al., 2019). NAP promotes chlorophyll degradation by upregulating ABA biosynthetic gene *AAO3*, and accelerates leaf senescence (Yang et al., 2014). Moreover, the ABA-NAP-SAG113 module controls stomatal movement and water loss during leaf senescence (Zhang and Gan, 2012; Zhang et al., 2012). In view of ABA actions in leaf senescence, it is thought that loss function of ABA biosynthetic genes should cause a delayed senescence. On the contrary, mutation in ABA2 (*aba2/eas1*) decreases ABA content but accelerates leaf senescence (Pourtau et al., 2004; Song et al., 2016). This opposite role may be explained by the role of ABA in both cytoprotective and senescence activities, and the function of ABA in influencing leaf yellowing is accurately balanced by both processes depending on plant age or environmental conditions.

In addition to the endogenous ABA content, ABA signaling pathway also plays essential roles in regulation of leaf senescence. As members of the receptors for ABA signaling, plants overexpressing P*YL8* and *PYL9* exhibit enhanced dark-induced or ABA-induced leaf senescence (Lee et al., 2015; Zhao et al., 2016). Correspondingly, *pyl duodecuple* mutant is extremely insensitive to ABA-induced leaf senescence (Zhao et al., 2018). Diverse TFs contribute to ABA-mediated leaf senescence. ABIG1 is induced by drought and ABA, and relays drought through ABA signal to promote leaf senescence (Liu et al., 2016). ABA-responsive element (ABRE)-binding TFs, ABF2/ABF3/ABF4 directly activate expression of chlorophyll catabolic enzyme genes (*NYE1*, *NYC1*, and *PAO*) and SAGs to mediate ABA-triggered leaf senescence and chlorophyll degradation (Gao et al., 2016). Similarly, OsNAP and ONAC054 which are induced by ABA promote the onset and progression of leaf senescence via positively regulating chlorophyll degradation and SAGs (Liang et al., 2014; Sakuraba et al., 2020), thus OsNAP and ONAC054 link ABA to leaf senescence by fine-tuning expression of SAGs. ABI5 acts as another core regulator in ABA-mediated leaf senescence. In apple, MdBBX22, MdWRKY40, and MdbZIP44 all interact with MdABI5 to delay or promote leaf senescence via repressing or enhancing its transcriptional activity (An et al., 2021b). The LEA protein, ABR, is also regulated by ABI5 involving in dark-induced leaf senescence (Su et al., 2016).

As a stress-responsive hormone, ABA mediates the environmental stress-induced leaf senescence process. *AtMYBL* substantially expresses in old leaves, and is also induced by ABA and salt stress. Overexpression of *AtMYBL* displays an enhanced leaf senescence with corresponding changes of chlorophyll content, ion leakage and SAGs expression (Zhang et al., 2011). As an ABA-induced transcription factor, NTL4 mediates drought-induced leaf senescence by promoting ROS production in *Arabidopsis* (Lee et al., 2012). VND-INTERACTING 2 (VNI2), an ABA-responsive NAC transcription factor, integrates ABA-associated abiotic stress signals into modulation of leaf longevity by regulating a subset of *COR* and *RD* genes (Yang et al., 2011). Additionally, ABA regulates leaf senescence by interplaying with other phytohormones. For example, ABA antagonizes CKs-delayed leaf senescence by upregulating the expression of *OsCKX11*, which catalyzes the degradation of CKs in senescing leaves (Zhang et al., 2021c). Collectively, ABA is a key regulator for integrating environmental stress signals into leaf senescence regulation, and is an important target for improving crop yield and quality through molecular breeding.

Brassinosteroids

Brassinosteroids (BRs) are a class of plant-specific steroid hormones, regulating many aspects of plant physiological processes, such as shoot, root, leaf development, and resistance to biotic stress (Peres et al., 2019). BRs accelerate leaf senescence in a dose-dependent manner (Saglam-Çag, 2007). The application of higher doses of exogenous epibrassinolide promotes leaf senescence, increases peroxidase activity and decreases chlorophyll content in wheat leaves. According to some studies on endogenous BR homeostasis, the stimulating effect of BR on leaf senescence was also elucidated. DET2 encodes a steroid 5α-reductase, and the delayed leaf senescence associated with an apparent phenotype of *det2* mutants in the brassinolide biosynthetic pathway may be due to the elimination of BR biosynthesis (Li et al., 1996). UGT73C6 was identified as an enzyme that catalyzes BR glucosylation and inactivates BR in the phytoplankton. Consistently, overexpression of *UGT73C6* delays leaf senescence (Husar et al., 2011). Transgenic plants overexpressing *P450su1*, which encodes the *CYP105A1* monooxygenase gene disrupt BR signaling by inactivating BRs, display the delayed senescence phenotype (Dasgupta et al., 2011). In addition, a dominant mutant *drl1-D* exhibited prolonged senescence as the endogenous levels of several BRs were significantly reduced (Zhu et al., 2013). The DRL1/BAT1 gene encodes an acyltransferase that catalyzes the conversion of BR intermediates to inactive conjugates via esterification. Transgenic creeping bentgrass overexpressing *AtBAT1* also showed delayed senescence (Han et al., 2017). These results suggest functional manipulation of BR levels to improve agronomic traits by regulating leaf senescence in dicot and monocot crops.

BR is perceived by leucine-rich repeat receptor kinase BRI1 (BRASSINOSTEROID INSENSITIVE 1) to induce signal transduction. The *bri1* exhibits a delay in leaf senescence, supporting a positive role of BR in senescence (Li and Chory, 1997). BES1 (BRI1 EMS SUPPRESSOR 1), a TF of BR signaling, accumulates in the nucleus in response to BR and accelerates leaf senescence once BES1 is activated (Yin et al., 2002). BRI1-associated kinase1 (BAK1), as a part of BR receptor complex, mediates BR-dependent responses. BAK1-LIKE 1 (BKK1) and

BAK7, the homologous of BAK1 are respectively reported to act redundantly with BAK1, however *bak1 bkk1* and loss function of both *bak1* and *bak7* display early senescence with upregulated SAGs (He et al., 2007; Jeong et al., 2010). These phenomena suggest that a BR-independent pathway involves in BAK1, BKK1, and BAK7-mediated senescence. Recently, ATBS1-INTERACTING FACTOR 2 (AIF2), a non-DNA-binding bHLH TF has been demonstrated to retard dark-triggered and BR-induced leaf senescence. BR-induced reduction of AIF2 protein associates with the promotion of leaf senescence (Kim et al., 2020). In summary, BR as an important growth-related hormone, its regulation of leaf senescence is always closely linked to the leaf development phenotype, so it may be integrated with other factors, similar to CK and auxin signaling to regulate age-induced or environmental stimulus-induced leaf senescence.

Strigolactones

Strigolactones (SLs) are a group of terpenoid lactones consisting of a tricyclic lactone and hydroxymethyl butanolide. SLs are well known as communication signals for parasitic and symbiotic interactions, and they were first identified to function as phytohormones to inhibit shoot branching in plants (Yamada and Umehara, 2015; Omoarelojie et al., 2019). The role of SLs in regulating plant growth and development was investigated, and the enhancement of leaf senescence by SLs was gradually elucidated. MAX3/RMS5/D17/DAD3 and MAX4/RMS1/D10/DAD1 encode carotenoid cleavage dioxygenases 7 (CCD7) and 8 (CCD8) respectively, involving in biosynthetic reactions of carlactone, the key precursor of SL. Interestingly, a reduction in expression of *CCD7* and *CCD8* results in delayed leaf senescence (Snowden et al., 2005; Ledger et al., 2010; Liu et al., 2013; Ueda and Kusaba, 2015), correlating with a positive role of SLs in senescence. *ORE9*, identical to *MAX2*, encoding an F-box protein of SL signal pathway, functions in degrading target proteins through ubiquitin-dependent proteolysis, and *ore9/max2* exhibits increased leaf longevity (Woo et al., 2001; Stirnberg et al., 2007). The orthologous of MAX2/ORE9 in rice, D3, disruption of which also delays leaf senescence with lower decrease of chlorophyll content and membrane ion leakage compared to wild type (Yan et al., 2007). A key characteristic of SLs regulation in leaf senescence is the coordination with other hormones and environmental cues. Leaf senescence was not affected by the application of GR24 (an artificial SL analog), however, once ethylene was present, GR24 strongly enhanced senescence (Ueda and Kusaba, 2015). *MAX3* and *MAX4* genes are drastically induced by ethylene, and SLs biosynthesis mutants such as *max1*, *max3*, and *max4* show a delayed senescence phenotype in the presence of ethylene (Ueda and Kusaba, 2015), indicating that ethylene mediates SLs biosynthesis during senescence. Furthermore, ENHANCED DISEASE RESISTANCE 1 (EDR1) mediates a phenotype of ethylene-induced senescence, which can be suppressed by mutation in ORE9/MAX2 (Tang et al., 2005). Therefore, this suggests that SLs are likely to accelerate leaf senescence following ethylene signaling. In numerous plants, the levels of endogenous SLs are elevated under conditions of phosphate deficiency (Yamada et al., 2014; Yamada and Umehara, 2015). In rice, SLs-deficient mutants were overly sensitive to GR24 application promoting leaf senescence when assessing chlorophyll content compared with adequate phosphate conditions. It was reported by Yamada et al. (2014) that SLs integrate with nutrient signals to regulate leaf senescence. The similar findings were described in the study that the addition of exogenous sugars alleviated SL-induced senescence in bamboo leaves under dark conditions (Tian et al., 2018). Analysis of transcription abundance in *max1* mutant during an extended night also deciphers a valuable association of carbon starvation and SLs signal in regulating leaf senescence (Xu et al., 2021). In conclusion, SLs are an important class of plant hormones that integrate multiple signals in the regulation of leaf senescence. Identification of more direct downstream targets of the SLs pathway, especially MAX2, is of great interest to elucidate the molecular mechanisms of SLs in leaf senescence.

PEPTIDE HORMONES THAT REGULATE LEAF SENESCENCE

Intercellular communication is important to coordinate the growth and developmental programs of multicellular organisms. In plants, classical phytohormones associated with small lipophilic compounds, such as auxin, CKs, GAs, ABA, and ethylene, greatly contribute to intercellular interactions involving different aspects of growth and development. In addition to classical hormones, researches show that multiple families of small polypeptide signaling molecules also play crucial roles in cell-to-cell interaction (Kende and Zeevaart, 1997; Matsubayashi and Sakagami, 2006). These secretory or non-secretory peptides regulate plant growth and development, and responses to environmental stresses, including defense responses, shoot meristem maintenance, root growth, leaf-shape regulation, nodule development, and organ abscission (Matsubayashi and Sakagami, 2006; Marmiroli and Maestri, 2014; Grienenberger and Fletcher, 2015). For examples, systemin induces production of proteinase inhibitors I and II, and plays obvious roles in systemic wound responses in distal leaves (Pearce et al., 1991; Lee and Howe, 2003). RALF (Rapid ALkalinization Factor) can cause alkalinization of the culture medium and a concomitant activation of an intracellular mitogen-activated protein kinase (Pearce et al., 2001). With the progress of researches, RALFs were unraveled to regulate myriad physiological processes, such as root growth and development, root hair size, pollen tube growth, polytubey block, salt stress, and so on (Pearce et al., 2001; Mecchia et al., 2017; Zhu et al., 2020; Zhao et al., 2021; Zhong et al., 2022). These results strengthen the importance of peptides' functions in plants. As the biological activities and functions of these peptide molecules are understood, they are considered to be "peptide hormones". However, the function of peptide hormones in leaf senescence is largely unknown.

Recently, several researches provide insight into the regulatory mechanism of peptide hormones in leaf senescence. The small secreted peptide CLE14 (CLAVATA3/ESR-RELATED 14) postpones leaf senescence by transcriptional activation of JUB1-dependent ROS scavenging genes (**Figure 1**), leading to reduced

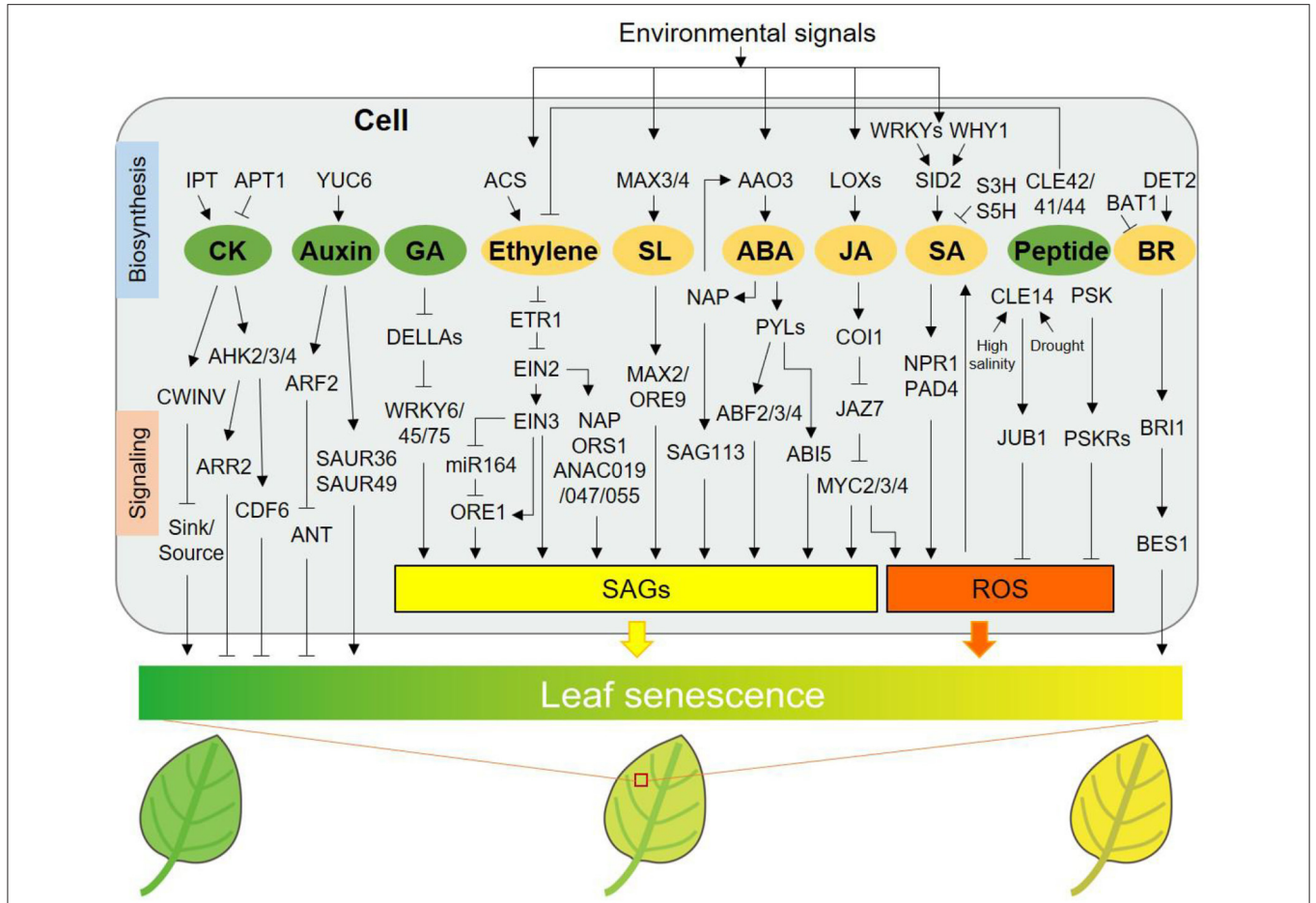

FIGURE 1 | Regulation of leaf senescence by classical and peptide hormones. IPT and APT1 participate in CK biosynthesis and catabolism, respectively. AHK2/3/4, ARR2, and CDF6, signaling components of CK, delay leaf senescence. CWINV (cell-wall invertase) delays senescence via regulating sink-source relations. YUC6 delays leaf senescence by increasing auxin biosynthesis, while ARF2 and SAUR36/49 promote leaf senescence by transmitting auxin signal. ANT, a downstream component of ARF2, postpones senescence phenotype. DELLA proteins delay processes of GA-induced leaf senescence via inhibiting functions of various WRKYs. ACS is involved in ethylene biosynthesis. ETR1, one receptor of ethylene signaling pathway, is involved in ethylene-induced leaf senescence. Ethylene promotes leaf senescence through EIN2-EIN3-miR164-ORE1 pathway or several EIN2 downstream components, including NAP, ORS1, and ANAC019/047/055. SL accelerates leaf senescence *via* functions of MAX2/ORE9. NAP can elevate ABA biosynthesis via inducing AAO3 expression, and ABA-NAP-SAG113 pathway promotes leaf senescence. The receptors of ABA, PYLs and the downstream TFs, ABF2/3/4 and ABI5 all promote ABA-triggered leaf senescence. LOXs promote leaf senescence by increasing JA content under stress conditions. JA promotes senescence via signaling pathway relating to COI1, JAZs, and MYC2/3/4, with increased expression of *SAGs* and enhanced ROS. WHY1 and several WRKYs promote SA content through elevating expression of SID2, a key synthase for SA biosynthesis. S3H and S5H catalyze SA to decline activated form of SA. SA promotes leaf senescence dependent on NPR1 and PAD4, associated with ROS. Peptide hormones including CLE42/41/44, CLE14, and PSK delay leaf senescence. CLE42/41/44 function redundantly to delay senescence via antagonizing with ethylene pathway. CLE14 is induced by high salinity and drought stresses and reduces ROS level via transcriptional activation of JUB1, a NAC TF. PSK may be perceived by its receptor PSKRs to contribute to ROS scavenging. In addition, GA, ethylene, SL, and ABA are also associated with regulation of a series of *SAGs* expression. DET2 contributes to BR biosynthesis and BAT1 inactivates BR. BR accelerates leaf senescence through signaling transduction involving positive regulators, BRI1 and BES1. Hormones including ethylene, SL, ABA, JA, and SA play significant roles in integrating environmental signals into the regulation of leaf senescence. Hormones presented in green ellipses including CK, auxin, GA, and mentioned peptides delay leaf senescence, whereas ethylene, SL, ABA, JA, SA, and BR in orange ellipses promote leaf senescence, according to phenotypic changes caused by exogenous application. SAGs, senescence-associated genes; ROS, reactive oxygen species.

ROS level in leaves (Zhang et al., 2022b). CLE14 is significantly induced by age, high salinity, drought, ABA, SA, and JA, thus it acts as a "brake signal" to modulate age-dependent and stress-induced leaf senescence (Zhang et al., 2022b). In another work, CLE42 delays leaf senescence by antagonizing ethylene signaling pathway. CLE42 suppresses ethylene biosynthesis and increases the accumulation of EBF proteins, sequentially resulting in the decreased function of EIN3. Additionally, CLE41/44 function redundantly with CLE42 to regulate leaf senescence (Zhang et al., 2022a). The peptides are usually recognized by membrane-localized receptors and transduce the signaling responses. It was reported that the CLE41/44, also called TDIF, could be bound by receptor TDR/PXY, a leucine rich repeat receptor-like kinase (LRR-RK) (Fisher and Turner, 2007; Hirakawa et al., 2008). With the help of co-receptor SERK, the TDIF-TDR/PXY signaling plays an important role in plant vascular development (Zhang

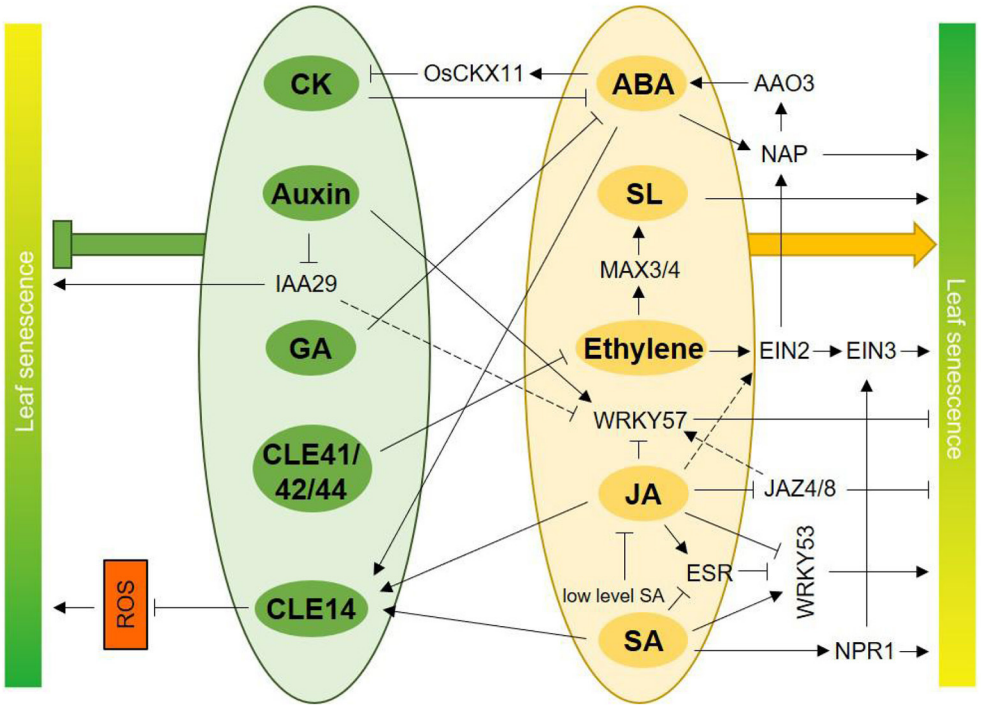

FIGURE 2 | Interplays among classic and peptide hormones in senescence regulation. CK inhibits ABA biogenesis and ABA represses CK content through elevating expression of *OsCKX11*. GA treatment inhibits the increase of ABA content during leaf senescence. *CLE14* is induced by ABA, JA, and SA, and CLE14 act as a "brake signal" to these hormones-induced leaf senescence though repressing ROS level. CLE41/42/44 delay leaf senescence by antagonizing with ethylene signaling pathway. Ethylene promotes SL biosynthesis via inducing expression of *MAX3* and *MAX4* during senescence, so ethylene and SL coordinately regulate leaf senescence. Phytohormone ABA and the core component of ethylene, EIN2, induce the expression of NAP, a positive regulator of leaf senescence. Conversely, NAP increases ABA biosynthesis through inducing expression of *AAO3*. JA-induced leaf senescence is dependent on EIN2. WRKY57 is a negative regulator of JA-induced leaf senescence. JA inhibits accumulation of WRKY57 protein and auxin promotes WRKY57, so that WRKY57 acts as an integrator of JA and auxin. Besides, the repressors of JA and auxin signaling pathway, JAZ4/8 and IAA29 both interact with WRKY57, which may be another regulatory level of interplay between JA and auxin. Ethylene and SA synergistically accelerate leaf senescence through interaction of NPR1 and EIN3 and a concomitant promotion of *SAGs* expression. SA represses the JA-inducible protein ESR, and ESR inhibits the functions of WRKY53, a positive regulator of leaf senescence. Furthermore, JA reduces the expression of *WRKY53* and SA induces *WRKY53* oppositely. Thus, ESR and WRKY53 are integrators of antagonism between JA and SA in leaf senescence. Moreover, low concentration of SA can alleviate JA-induced leaf senescence. Generally, hormones presented in green ellipses including CK, auxin, GA, CLE41/42/44, and CLE14 delay leaf senescence (bold green symbol). Hormones in orange ellipses including ABA, SL, ethylene, JA, and SA promote leaf senescence (bold orange arrow).

et al., 2016). Whether these receptors also take part in leaf senescence regulation is an interesting question for investigation. Especially, beyond the role of CLE41/CLE44 themselves in senescence, the TDIF-TDR/PXY can lead to inactivation of BES1 TF, a crucial regulator of senescence-promotion hormone BR, through regulation of GSKs activity (Kondo et al., 2014). The known CLE receptors are limited, it is necessary to find more CLE receptors for better understanding their functions in leaf senescence. In addition to CLE peptides, PSK (Phytosulfokine) and PSY1 are also involved in the regulation of leaf senescence. The membrane-localized PSKR is the receptor of PSK, and loss-of-function *pskr1-1* mutant exhibits premature leaf senescence, which is consistent with a delayed effect of exogenous PSK-α on senescence (Yamakawa et al., 1999; Matsubayashi et al., 2006). Interestingly, two other homologs of PSKR also encode LRR-RKs, one of which acts on PSY1 perception and has an overlapping function with PSKR; thus, the triple mutant shows an enhanced senescence phenotype compared to the single *pskr1* mutant (Amano et al., 2007). Posttranslational modification of small peptides is essential for their biological functions (Matsubayashi, 2014). Biological activities of PSK and PSY1 need tyrosine sulfation, catalyzed by transmembrane tyrosylprotein sulfotransferase (TPST). Interestingly, loss-of-function of TPST accelerates leaf senescence (Komori et al., 2009). PSK may influence leaf senescence through protective action in chlorophyll content under heat stress (Yamakawa et al., 1999), or delays senescence by elevating expression of ROS scavenging genes and reducing endogenous H_2O_2 accumulation during storage of fruits and flowers (Aghdam et al., 2021a,b). In summary, peptide hormones interact with classical hormones to regulate leaf senescence. Hundreds of peptide hormones and putative peptide molecules have been identified in plants, and their functions in the regulation of leaf senescence deserve further exploration.

CONCLUSIONS AND PERSPECTIVES

Leaf senescence is a highly coordinated process controlled by a complex network of genes. The classical plant hormones

and peptide hormones contribute significantly to the regulation of the initiation and progression of leaf senescence (**Figure 1**), which underlies the orderly degradation and macromolecular degradation of chloroplasts and is closely linked to the maximization of nutrient utilization for growth and development. Hormones can precisely regulate leaf senescence, thanks to the flexibility of their action. CKs, auxins and GA are known to delay leaf senescence, while ethylene, SA, JA, ABA, BRs and SLs promote senescence, and even peptides can regulate senescence in a viable manner. However, when referring to the role of a certain hormone in the regulation of leaf senescence, we must be aware of the dosage effect. Hormones at low concentrations delay leaf senescence, but high concentrations promote senescence (Song et al., 2016). This is partly attributed to the interactions between hormones (Saglam-Çag, 2007). Hormone-mediated leaf senescence involves signal transduction pathways and a myriad of transcriptional regulations, yet an increasing number of studies have reported multi-level gene regulation of leaf senescence. For example, post-transcriptional alternative splicing regulation of ONAC054 involves in ABA-induced leaf senescence (Sakuraba et al., 2020). Although ONAC054α is only induced by ABA, its alternative splicing form ONAC054β is induced by ABA and high concentration of ACC, thus the multilayered regulation provides more available regulatory nodes for hormonal interplays. The interaction between plant hormones is another advantage of their precise regulation of leaf senescence under different environmental conditions, which is crucial for the operation of agronomic improvement. The interplays among classic and peptide hormones in the regulation of leaf senescence was summarized in **Figure 2**. Understanding the functions of key genes or proteins that link different hormone signals will help us to understand the intrinsic regulation of leaf senescence by hormone signaling networks. In addition, hormones play an important role in integrating environmental signals into specific components or pathways associated with leaf senescence. In conclusion, dissecting the novel functions of hormone signaling components in leaf senescence is worth further attention.

The integration of plant hormones affecting the process of leaf senescence and environmental factors is more easily achieved in traditional experimental systems. Nevertheless, the initiation of leaf senescence depends on age-related factors, which is a major part of the mystery explored. Although hormones, for instance ethylene, cannot affect leaf senescence until leaf age reaches a certain developmental stage (Jing et al., 2002), they ensure the regulation of plant growth and development, which may contribute to the accumulation of age factors that alter the onset of aging. The initiation of senescence is not uniform, as leaf cells are usually in different states of senescence over a certain period of time. Taking advantage of single-cell sequencing, combined with spatiotemporal transcriptome analysis, offers the possibility to address the challenges of senescence research, leading to a better understanding of the initiation of senescence and the corresponding hormonal functions during this process.

Peptide hormones consist of a family of different classes of peptides that regulate plant growth, development and response to environmental stresses. So far, CLEs and PSK have been described as substances that regulate leaf senescence (Matsubayashi et al., 2006; Zhang et al., 2022a), and more peptide signaling molecules deserve to be explored to expand the understanding of leaf senescence. LRR-RKs work redundantly in perception of PSK and PSY1, and sometimes one LRR-RK does not only recognize one particular peptide (Amano et al., 2007), suggesting difficulties in finding novel peptide components and establishing explicit signaling transduction. Distinct from forward genetic screening and reverse genetic analysis, chemical genetic screening can overcome the problem of gene functional redundancy and is a good option to further explore more components of leaf senescence. With the establishment of more complex hormonal signaling pathways associated with leaf senescence, we expect that the regulatory mechanisms of leaf senescence will provide important clues for improving crop yield and quality.

AUTHOR CONTRIBUTIONS

ZL, HG, and PH conceived and designed this review. PH wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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The INFLORESCENCE DEFICIENT IN ABSCISSION-LIKE6 Peptide Functions as a Positive Modulator of Leaf Senescence in *Arabidopsis thaliana*

Cun Guo[1,2†], Xiaoxu Li[3†], Zenglin Zhang[1], Qi Wang[1], Zhenbiao Zhang[1,2], Lichao Wen[1,2], Cheng Liu[4], Zhichao Deng[1,2], Yumeng Chu[1], Tao Liu[1,2] and Yongfeng Guo[1]*

[1]Chinese Academy of Agricultural Sciences, Tobacco Research Institute, Qingdao, China, [2]Graduate School of Chinese Academy of Agricultural Sciences, Beijing, China, [3]Technology Center, China Tobacco Hunan Industrial Co., Ltd., Changsha, China, [4]QuJing Tobacco Company, Qujing, China

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Shaanxi Normal University, China

***Correspondence:**
Yongfeng Guo
guoyongfeng@caas.cn

[†]*These authors have contributed equally to this work*

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Leaf senescence is a highly coordinated process and has a significant impact on agriculture. Plant peptides are known to act as important cell-to-cell communication signals that are involved in multiple biological processes such as development and stress responses. However, very limited number of peptides has been reported to be associated with leaf senescence. Here, we report the characterization of the INFLORESCENCE DEFICIENT IN ABSCISSION-LIKE6 (IDL6) peptide as a regulator of leaf senescence. The expression of *IDL6* was up-regulated in senescing leaves. Exogenous application of synthetic IDL6 peptides accelerated the process of leaf senescence. The *idl6* mutant plants showed delayed natural leaf senescence as well as senescence included by darkness, indicating a regulatory role of IDL6 peptides in leaf senescence. The role of IDL6 as a positive regulator of leaf senescence was further supported by the results of overexpression analysis and complementation test. Transcriptome analysis revealed differential expression of phytohormone-responsive genes in *idl6* mutant plants. Further analysis indicated that altered expression of IDL6 led to changes in leaf senescence phenotypes induced by ABA and ethylene treatments. The results from this study suggest that the IDL6 peptide positively regulates leaf senescence in *Arabidopsis thaliana*.

Keywords: leaf senescence, IDL6, plant peptide, *Arabidopsis*, transcriptome analysis, phytohormone

INTRODUCTION

As a vital part in plants' life cycle, leaf senescence is a type of post-mitotic senescence which involves a strictly programmed cell death process (Gan, 2003). During leaf senescence, cellular organelles and macromolecules are degraded and nutrients are remobilized to reproductive organs and new tissues (Li et al., 2017; Guo et al., 2021). In agricultural applications, artificially accelerating or delaying leaf senescence in crop plants could achieve higher yields and better quality (Gan, 2014; Guo and Gan, 2014; Woo et al., 2019). During the past two decades, a large number of stay-green loci and senescence regulators have been identified from model

plant systems which can be potentially used in manipulating leaf senescence for crop improvement (Vadez et al., 2011; Guo et al., 2015; Del Pozo et al., 2016).

Leaf senescence is an extremely complicated process and can be triggered by diverse factors including internal and external factors (Guo and Gan, 2005). The former includes phytohormones and reproduction growth (Lim et al., 2007). For instance, abscisic acid (ABA; He et al., 2005), ethylene (Iqbal et al., 2017), Jasmonate (JA; Hu et al., 2017) and salicylic acid (SA; Abreu and Munné-Bosch, 2008) accelerate leaf senescence, while cytokinin (CK; Hwang et al., 2012), auxin (IAA; Lim et al., 2010) and gibberellins (GA; Rodrigues et al., 2012) are negative regulators of senescence. External senescence-regulating factors are complex and generally associated with biotic/abiotic stresses, such as darkness, salinity, drought, extreme temperature and pathogen infection. Interestingly, some genes involved in biotic/abiotic stresses have also been reported to regulate leaf senescence. *WRKY55* was reported to be involved in both leaf senescence and disease resistance by regulating the accumulation of reactive oxygen species (ROS) and SA (Wang et al., 2020). In rice, *ONAC106* can be induced by salt stress, and functions as a negative regulator of leaf senescence (Sakuraba et al., 2015).

As an important part of cell-to-cell interaction in higher plants, peptide signals have been characterized to be involved in various aspects of plants' life cycle including meristem organization (Guo et al., 2015), self-incompatibility (Liu et al., 2021), reproduction (Kim et al., 2021), organ abscission (Jinn et al., 2000), root growth (Huang et al., 2006), stress responses (Yamaguchi et al., 2010), hormone signaling (Estornell et al., 2013), nodule development (Mohd-Radzman et al., 2016) and RNA metabolism (Matsubayashi and Sakagami, 2006; Zhang et al., 2020). INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and IDA-Like (IDL) peptides are a small subgroup of plant peptides with 9 members in *Arabidopsis*, which possess an N-terminal signal peptide and a C-terminal extended PIP (EPIP) domain (Stenvik et al., 2008a,b). Some IDL peptides have been characterized for their roles in cell separation and stress responses. The IDA peptide was identified to control floral organ abscission and lateral root emergence *via* interacting with its receptors HAESA (HAE) and HAESA-LIKE2 (Stenvik et al., 2008b; Kumpf et al., 2013; Liu et al., 2013). The IDA-HAE/HSL2 signaling module functions in activating the mitogen-activated protein (MAP) kinase cascades, which in turn regulate the expression of cell wall-modifying and hydrolytic enzymes (Kumpf et al., 2013; Meng et al., 2016). In addition, SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1) has been shown to act as a co-receptor of the IDA peptide in regulating flower abscission (Santiago et al., 2016). The coding genes of IDL6 and IDL7 peptides were shown to be induced rapidly by various stresses and have been suggested to be negative modulators of stress-induced ROS signaling (Vie et al., 2015, 2017). A recent study showed that the IDL6-HAE/HSL2 signaling module functions in facilitating infection of *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 by promoting pectin degradation in *Arabidopsis* leaves (Wang et al., 2017).

A recent study reported that the CLAVATA3/ESR-RELATED 14 (CLE14) peptide serves as a senescence-regulating signal in *Arabidopsis* (Zhang et al., 2021), raising the possibility of more peptide signals involved in leaf senescence. Here we describe the characterization of the IDL6 peptide in regulating leaf senescence of *Arabidopsis*. The expression of *IDL6* was detected to be up-regulated in senescing leaves. The loss-of-function *idl6* mutant displayed a delayed senescence phenotype, and this phenotype could be rescued by the *IDL6* gene. Overexpressing *IDL6* or exogenous application of synthetic IDL6 peptides accelerated leaf senescence. Transcriptome analysis showed differential expression of phytohormones-responsive genes in *idl6* mutant plants. Further test of ABA and ethylene-induced senescence on detached leaves suggest that IDL6 might function *via* affecting ABA and ethylene signaling. Taken together, the results from this study indicate that IDL6 is a positive modulator of leaf senescence in *Arabidopsis*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis Columbia ecotype (Col-0) was used as the wild type in this study. The *idl6* mutant (SALK_074245) was obtained from the *Arabidopsis* Biological Resource Center (ABRC) and genotyped *via* PCR. The transcript abundance of the *IDL6* gene in *idl6* and Col-0 plants was detected by quantitative real-time PCR (qRT-PCR).

Arabidopsis seeds were surface sterilized with 70% (v/v) ethanol for 5 min and spread evenly in half-strength Murashige and Skoog (1/2 MS) media. Then the media were cultivated at 23°C with continuous light after being placed in a 4°C refrigerator for 2 days. Two weeks later, seedlings were transplanted to soil mixture, and kept in a growth chamber at 23°C with continuous light (Li et al., 2021; Zhang et al., 2021). Leaves at different development stages were selected for gene expression analysis. The fifth and sixth rosette leaves at different developmental stages were collected to explore the expression pattern of *IDL6* (YL, young leaves, 4-week-old; NS, non-senescence, 4.5-week-old; ES, early senescence, 5.5-week-old, LS, late senescence, 6.5-week-old). To this end, leaf samples of three biological replicates were collected and frozen in liquid nitrogen for RNA extraction.

Generation of Constructs and Transgenic Plants

For overexpression analysis, *IDL6* (AT5G05300) CDS was PCR amplified from cDNA of Col-0 leaves. The PCR product was purified and inserted into the enzyme digested pCHF3 vector with Sac I by Infusion (Clontech, Beijing, China). The promoter sequence of *IDL6* was amplified from genomic DNA of Col-0 leaves and the PCR product was purified and inserted into the enzyme digested pBI121a vector (modified from pBI121) with Sac I by Infusion. Similarly, the promoter plus CDS fragment of *IDL6* was cloned into pZP211 for complementation test. All constructs were confirmed by Sanger sequencing and transformed into *Agrobacterium* competent cells (GV3101), which were used to transform *Arabidopsis* by *Agrobacterium*-mediated floral dip method (Zhang et al., 2006). The positive transgenic plants were screened on 1/2 MS medium containing 50 mg/l kanamycin and T3 homozygous lines were used for further study.

RNA Extraction and qRT-PCR

Total RNAs from each sample were extracted by using the Ultrapure RNA Kit (cwbiotech, Beijing, China). Reverse transcriptions were performed using the Evo M-MLV Mix Kit with gDNA Clean for qPCR (Accurate Biotechnology, Changsha, China). qRT-PCR was performed using a Roche LightCycler 480 Real-Time PCR instrument with SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology, Changsha, China). *ACT2* was used as an internal control and all experimental data were obtained with three technical repetitions. The resulted Data were analyzed *via* the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All primers used in this study are listed in **Supplementary Table 1**.

Determination of Fv/Fm, Chlorophyll Content and Ion Leakage

The chlorophyll fluorescence Fv/Fm of individual leaves was determined using the IMAGING-PAM Mseries Chlorophyll Fluorescence System (LI-6400-40 LCF, Walz, Effeltrich, Germany) according to the manufacturer's instructions (Rossel et al., 2006). For determination of chlorophyll content, 100% methanol was used in dissolving chlorophyll from leaves. After chlorophyll was completely released, absorbance at 666 and 653 nm was obtained with a spectrophotometer (ClarioSTAR, BMG LABTECH, Offenburg, Germany), chlorophyll content was calculated as previously described (Lightenthaler, 1987). For ion leakage measurement, leaves were immersed in deionized distilled water, shaken at 25°C for 30 min, and the beginning conductivity was measured using a digital conductivity meter (Thermo Fisher Scientific Traceable, Hampton, NH, United States of America). The samples were then boiled for 15 min and then the second conductivity was measured. The percentage of the first measurement over the second measurement was used as the membrane leakage indicator (Zhao et al., 2018).

Peptide Synthesis and Detached Leaf Senescence Assay

The IDL6 peptide EPIP-domain (**Supplementary Figure 1**) sequence FGSLVLNALPKGSVPASGPSKRIN was synthesized by Genscript (Nanjing, China) at the purity of 95%. For detached leaf senescence assay, the seventh and eighth leaves of 8-week-old wild-type plants were excised and incubated in plates containing 1 μM IDL6 peptides. Leaf senescence phenotypes were recorded and chlorophyll contents were measured.

Dark- and Phytohormone-Induced Leaf Senescence

All plants were grown on soil in a growth chamber under continuous light at 23°C. For dark-induced leaf senescence analysis, the fifth, sixth and seventh leaves of 4.5-week-old wild-type, *idl6* mutant, overexpression and rescued lines were excised and placed onto moistened filter paper inside foil-wrapped petri dishes as described previously (Li et al., 2016). Pictures were taken 0 d, 3 d, and 5 d after treatments. For hormone-induced senescence treatments, the seventh, eighth and nineth leaves of 6-week-old plants were incubated in liquid 1/2 MS media with or without 10 μM ABA or 50 μM ethephon, respectively, as described previously (Li et al., 2021). Pictures were taken at 0 d, 1 d, 3 d, and 5 d after treatments. For all measurements, three biological replicates were performed.

RNA-Sequencing Analysis

The sixth and seventh leaves of 4-week-old Col-0 and *idl6* plants were collected and frozen in liquid nitrogen immediately. Each plant sample was represented by three biological replicates. The samples were entrusted to Shanghai OE Biotech for RNA-Seq. The quality of the sequencing data was scrutinized in terms of total raw reads, total clean reads, Q20 percentage, and GC percentage. DEGs were filtered using the following criteria: $|\text{Log2 (fold change)}| > 2.0$, $p < 0.05$. KEGG enrichment analysis was based on the path entries with the number of corresponding differential genes greater than 2, and sorted according to the corresponding -log10 *p*-value. Raw RNA-seq reads are available at the National Center for Biotechnology Information (BioProject ID: PRJNA821657).

Statistical Analysis

All data analyses in this study were performed based on at least three biological replicates. Statistically significant differences were determined using Student's *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Values in graphs are the mean value ± SE of all replicates.

RESULTS

ILD6 Is Up-Regulated During Leaf Senescence

The 5th and 6th rosette leaves of *Arabidopsis* (Col-0) plants were collected to explore the expression pattern of *IDL6* at four different developmental stages, including young leaf (YL), non-senescence leaf (NS), early senescence leaf (ES) and late senescence leaf (LS; **Figure 1A**). Chlorophyll contents decreased from NS to LS stage, indicating progression of leaf senescence (**Figure 1B**). As expected (Gan and Amasino, 1995; Zhang et al., 2012; Guo et al., 2021), the rubisco small subunit encoding gene *RBCS* was down-regulated during leaf senescence (**Figure 1C**), while the senescence marker gene *SAG12* was up-regulated from NS stage to LS stage, and was highly expressed at the late senescence stage (**Figure 1D**). The *IDL6* transcripts were detected to be highly expressed in leaves at both early and late senescence stages (**Figure 1E**).

Considering that natural senescence of a single leaf proceeds from the tip to the base part, an early senescence leaf from the 6th position of 8-week-old wild-type *Arabidopsis* was collected for analysis. From this leaf, three sections were isolated, including the tip, the middle and the base (**Figure 2A**). As expected, chlorophyll contents and photosynthetic rates declined gradually from tip to base (**Figures 2B,C**). Additionally, the transcript abundance of *SAG12* and *RBCS* were correlated with the degree of leaf senescence, with the highest expression levels of *RBCS* and *SAG12* detected in the base and the tip part, respectively, (**Figures 2E,F**). *IDL6* gene was expressed similarly to *SAG12*, which increased continuously from the leaf base

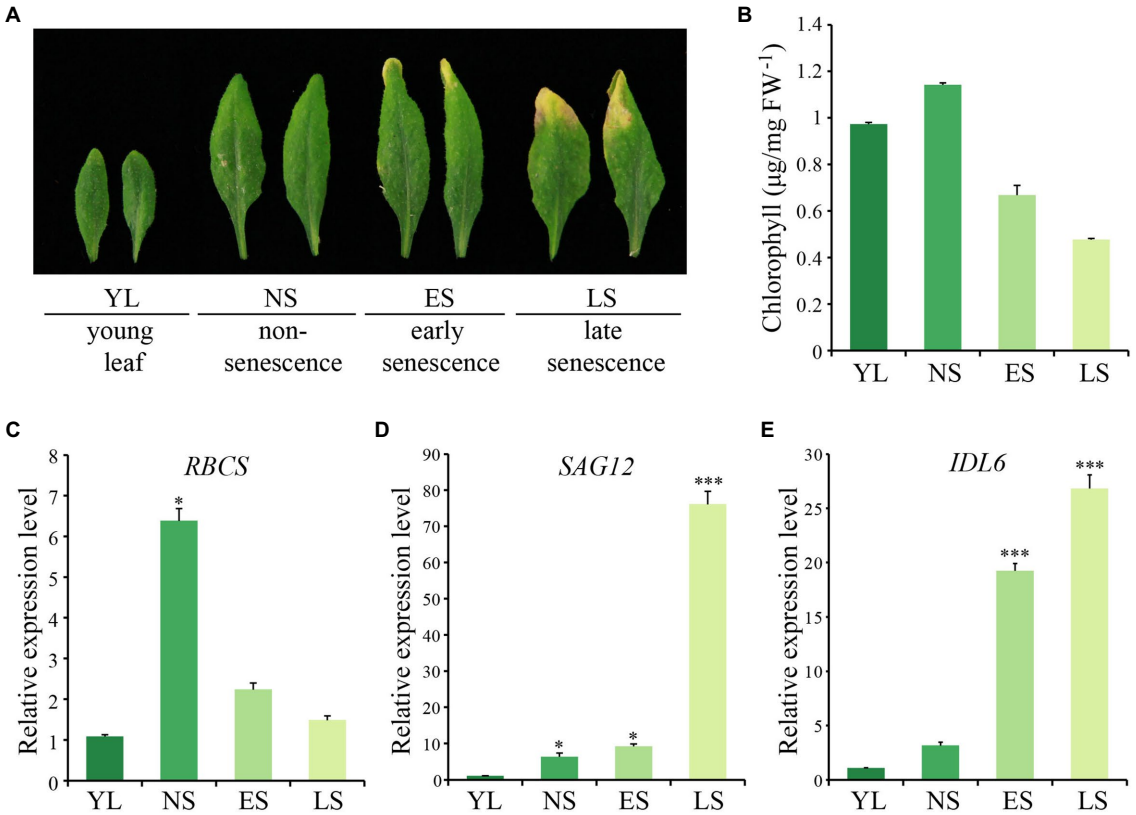

FIGURE 1 | The expression pattern of *IDL6* during leaf senescence. **(A)** The *Arabidopsis* wild-type leaves at different developmental stages. YL: young leaf, NS: non-senescence leaf, ES: early senescence leaf, LS: late senescence leaf. **(B)** The chlorophyll contents of leaves at different developmental stages. **(C–E)** The transcript abundance of *RBCS*, *SAG12* and *IDL6* during leaf senescence. The data are means ± SD of three biological repeats. Significant difference compared with the YL was determined by Student's *t*-test (*$p < 0.05$ and ***$p < 0.001$).

to the tip and was highly expressed in the senescent leaf tip (**Figure 2G**). Furthermore, the *IDL6* promoter was cloned in front of the *b-glucuronidase* (*GUS*) gene and used for transforming wild-type plants. When *ProIDL6::GUS* leaves at the early senescence stage were used for GUS staining, more GUS signals were detected at the tip of leaves. Notably, strong GUS activity was observed at the base part of mechanically damaged leaves, suggesting that *IDL6* was also induced by wounding (**Figure 2D**).

Exogenous Application of Synthetic IDL6 Peptides Accelerates Leaf Senescence

As a special group of plant hormones, peptide ligands could be artificially synthesized to explore their function. In previous studies, the EPIP-domain (extended PIP) of AtIDA and MiIDL1 were synthesized in determining their roles in floral organ shedding (Stenvik et al., 2008b; Kim et al., 2018). The EPIP sequence of IDL6 peptide was confirmed by sequence alignment according to previous studies (Butenko et al., 2003; Stenvik et al., 2008b) and was artificially synthesized for treating detached leaves (**Supplementary Figure 1**). Leaf disks of the seventh and eighth leaves from 8-week-old wild-type plants were treated with 1 μM IDL6 EPIP peptides. Three and 4 days later, an early senescence phenotype can be observed on the leaf disks treated with IDL6 EPIP peptides compared to mock treatments (**Figure 3A**). In consistent with visible phenotypes, significant decline of chlorophyll content and Fv/Fm was detected in leaves treated with IDL6 EPIP peptides in comparison with mock treatments (**Figures 3B,C**). Besides, the *RBCS* expression level of mock treatments was higher than peptide treatments, while, the *SAG12* expression level of mock treatments was lower than peptide treatments (**Figures 3D,E**). This result suggests that the IDL6 EPIP peptide functions in accelerating leaf senescence.

Loss of *IDL6* Function Delays Leaf Senescence

To confirm the role of IDL6 in regulating of leaf senescence, a mutant line SALK_074245 (*idl6*) with a T-DNA insertion before the start codon of the *IDL6* gene was obtained (**Figure 4A**). PCR and Sanger sequencing were performed to identify homozygous *idl6* mutant plants. *IDL6* transcript was not detected in the late senescence leaf of *idl6* plants, suggesting that is *idl6* a null mutant (**Figures 4B,C**). Two rescue lines were obtained by transforming the *IDL6* gene back to *idl6* mutant plants (**Figure 4C**; **Supplementary Figure 2**).

No obvious phenotypic changes were found between the *idl6* mutant line, wild-type and rescue lines at early developmental

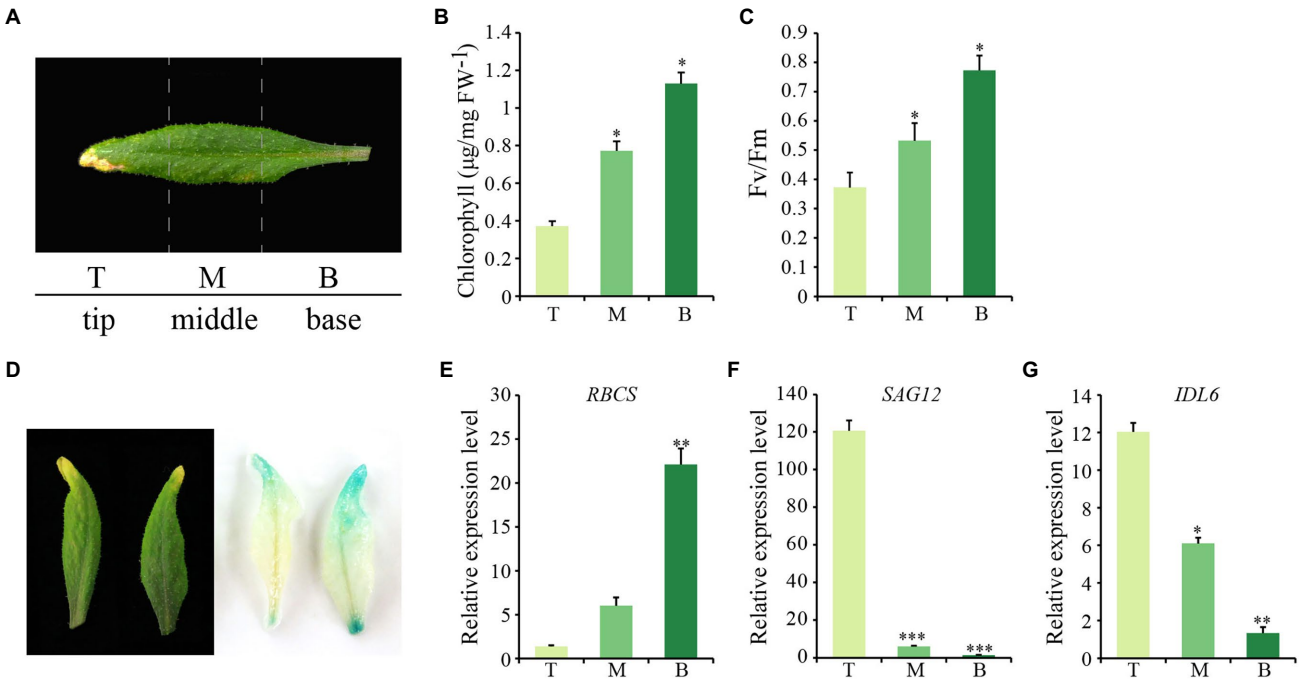

FIGURE 2 | The expression of *IDL6* in different parts of a senescing leaf. **(A)** Representative image of three sections of *Arabidopsis* wild-type leaves. T: leaf tip, M: middle part, **B**: leaf base. Chlorophyll contents **(B)** and photosynthetic rates **(C)** in different leaf sections. **(D)**: GUS staining on 7th and 8th rosette leaves from 8-week-old *ProIDL6*::*GUS* transgenic line. **(E-G)** Transcript abundance of *RBCS*, *SAG12* and *IDL6* in different leaf sections. The data are means ± SD of three biological repeats. Significant differences compared with the tip part were determined by Student's *t*-test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

stages. At alter stage, when 8-week-old plants were compared, the loss-of-function *idl6* mutant displayed a significant delay in leaf senescence. The delayed senescence phenotype of *idl6* plants was rescued to wild type in plants of the rescue lines (**Figures 4D,E**). To further characterize the senescence phenotypes of the *idl6* mutant, the 12 rosette leaves from plants of different genotypes were divided into four groups to collect physiological data (**Figure 4E**). As a result, the highest Fv/Fm ratio and chlorophyll contents were found in leaves of each group from the *idl6* mutant (**Figures 4F,G**). Ion leakage is an important plasma membrane integrity indicator, and is considered one of the most important indicators of leaf senescence (Feller and Fischer, 1994). The leaves of the *idl6* mutant showed significantly lower ion leakage rates compared with the wild-type and rescue lines (**Figure 4H**). As expected, the *RBCS* expression level of *idl6* plants was higher than that of the wild-type and complementary lines, while the *SAG12* expression level of the *idl6* mutant was lower than wild-type and complementary lines (**Figures 4I,J**).

Overexpression of *IDL6* Accelerates Leaf Senescence

To further explore the function of IDL6 in leaf senescence, the *IDL6* gene was overexpressed using the 35S promoter. Two overexpression lines (namely *OE-1* and *OE-4*) were selected to for further analysis. The results showed that leaves of these overexpression lines exhibit morphologies of smaller size, which is consistent with what Wang et al. (2017) reported. The IDL6-OE lines displayed an early leaf senescence phenotype compared with wild type (**Figures 5A–C**). The chlorophyll contents and Fv/Fm values in the overexpression lines were significantly lower than that of wild-type plants (**Figures 5D,E**). The high ion leakage in the overexpression lines also suggested that the leaf senescence progression was accelerated with enhanced *IDL6* expression (**Figure 5F**). This was also demonstrated by the expression of *RBCS* and *SAG12* in wild-type and overexpressed plants (**Figures 5G,H**).

Transcriptome Analysis Reveals the Importance of Hormone Signaling in IDL6-Mediated Leaf Senescence

Earlier studies suggested that 10–16% of all genes show differential expression during leaf senescence (Breeze et al., 2011; Woo et al., 2016). To explore gene expression changes caused by the *idl6* mutation, the 6th and 7th leaves with similar degree of yellowing from wild-type and *idl6* plants were collected to perform RNA-seq analysis.

The transcriptome analysis identified 2,618 differentially expressed genes (DEGs) in *idl6* mutant compared with wild type (**Figure 6A; Supplementary Table 2**). Interestingly, The WRKY transcription factor *WRKY53* (Miao et al., 2004) and Dof transcription factor *CDF4* (Xu et al., 2020), which have been reported to be positive regulators of leaf senescence, were significantly down-regulated in the *idl6* mutant

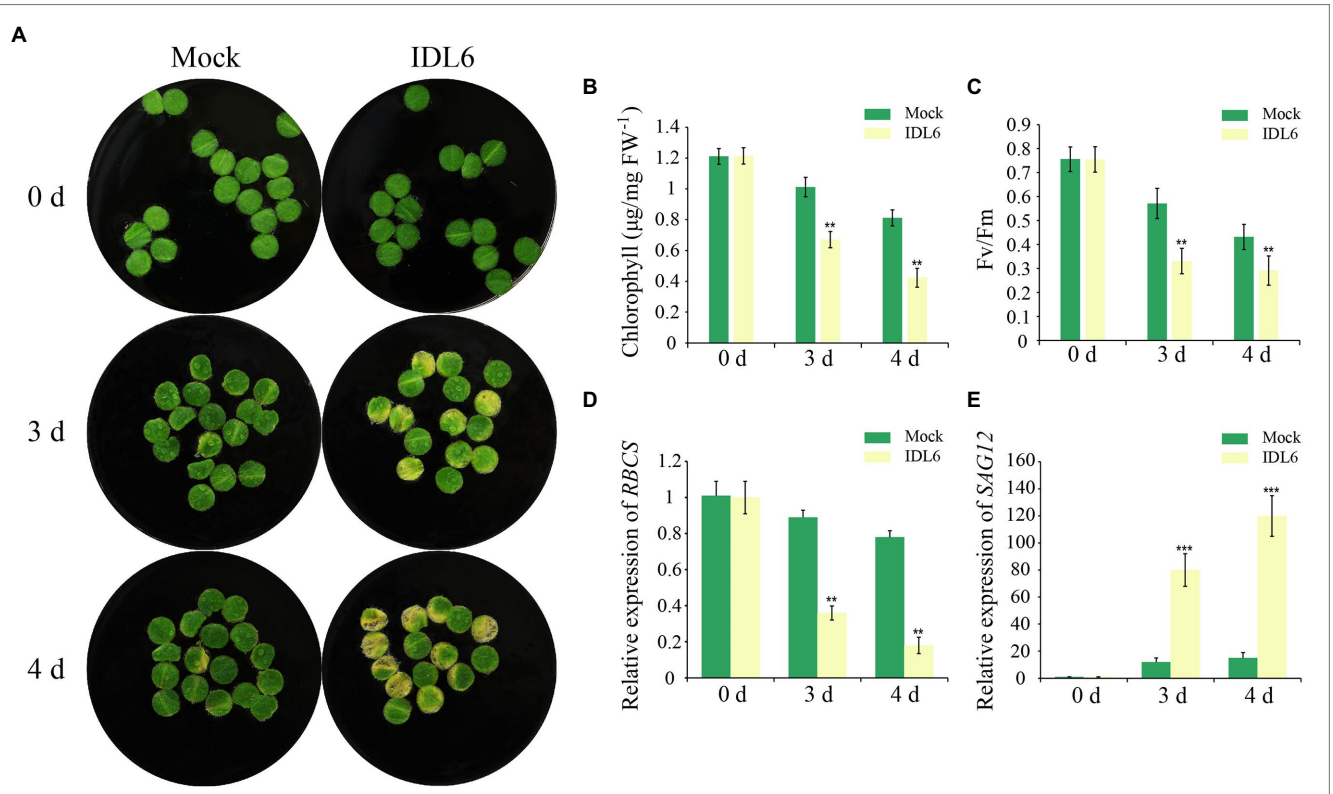

FIGURE 3 | The senescence phenotypes of detached leaves treated with synthetic IDL6 peptides. **(A)** The detached leaves from wild type of *Arabidopsis* exhibited early senescence under IDL6 EPIP peptide treatments. Leaves were kept in a growth chamber at 23°C with continuous light. Changes in chlorophyll content **(B)** and photosynthetic rate **(C)** in the treated leaves. The relative expression of *RBCS* **(A)** and *SAG12* **(E)** in leaves from each group. Error bars indicate the SE ($n > 10$). Significant difference compared with the Mock was determined by Student's *t*-test (**$p < 0.01$ and ***$p < 0.001$).

(**Figure 6C**). Moreover, a number of stress responsive WRKY transcription factors, including *WRKY38* and *WRKY62* (Kim et al., 2008), were significantly down-regulated in *idl6* leaves (**Figure 6C**), Also, the expression levels of these genes were confirmed in wild-type, *idl6*, rescue and overexpression lines with qRT-PCR (**Supplementary Figure 3**).

When KEGG analyses of DEGs were performed to compare wild-type and the *idl6* mutant, more DEGs were enriched in plant hormone signaling pathways (**Figure 6B**). The ABA-INSENSITIVE1 (ABI1) protein phosphatase 2C and ABI2 were reported to be negative regulators of ABA signaling (Merlot et al., 2001). Both *ABI1* and *ABI2* were up-regulated in *idl6* mutant (**Figure 6D**). In addition, a number of genes related to ABA signal transduction, including *ABI5*, *PYL5*, *PYL4*, *OST1*, *ABF2* and *ABF4* (Finkelstein and Lynch, 2000; Mustilli et al., 2002; Kim et al., 2004; Santiago et al., 2009) showed differential expression in *idl6* plants (**Figure 6D**). These results suggest that IDL6 might be involved in hormonal-induced leaf senescence.

IDL6 Might Be Involved in ABA- and Ethylene-Induced Leaf Senescence

A growing body of evidence suggests that both ABA and ethylene are positive regulators of leaf senescence. In order to find out whether IDL6 is involved in leaf senescence induced by ABA or ethylene, the seventh, eighth and nineth leaves of wild-type, *idl6*, *IDL6-OE1* and *IDL6-RE4* plants were isolated and treated with 10 μm ABA or 50 μm ethylene. As expected, leaf senescence was accelerated by ABA and ethylene treatments. Significant leaf yellowing was observed on leaves from wild type 1 day after ABA treatments and 3 days after ethylene treatments. Leaves from the *idl6* mutant showed delayed while leaves from the *IDL6* overexpression line *OE1* showed accelerated senescence compared with wild type. The *IDL6* rescue-line showed the similar leaf senescence progress with wild type after both ABA and ethylene treatments (**Figures 7A–C**). Changes in photosynthetic rates and chlorophyll contents in the treated leaves were consistent with the leaf yellowing progression (**Figures 7D,E**). These results hint that IDL6 might be involved in ABA- and ethylene-induced leaf senescence.

IDL6 Functions in Promoting Dark-Induced Senescence

To further explore the role of IDL6 peptides in leaf senescence, dark treatments were performed to induce leaf senescence. The 5th, 6th, and 7th leaves were excised from 4.5-week-old

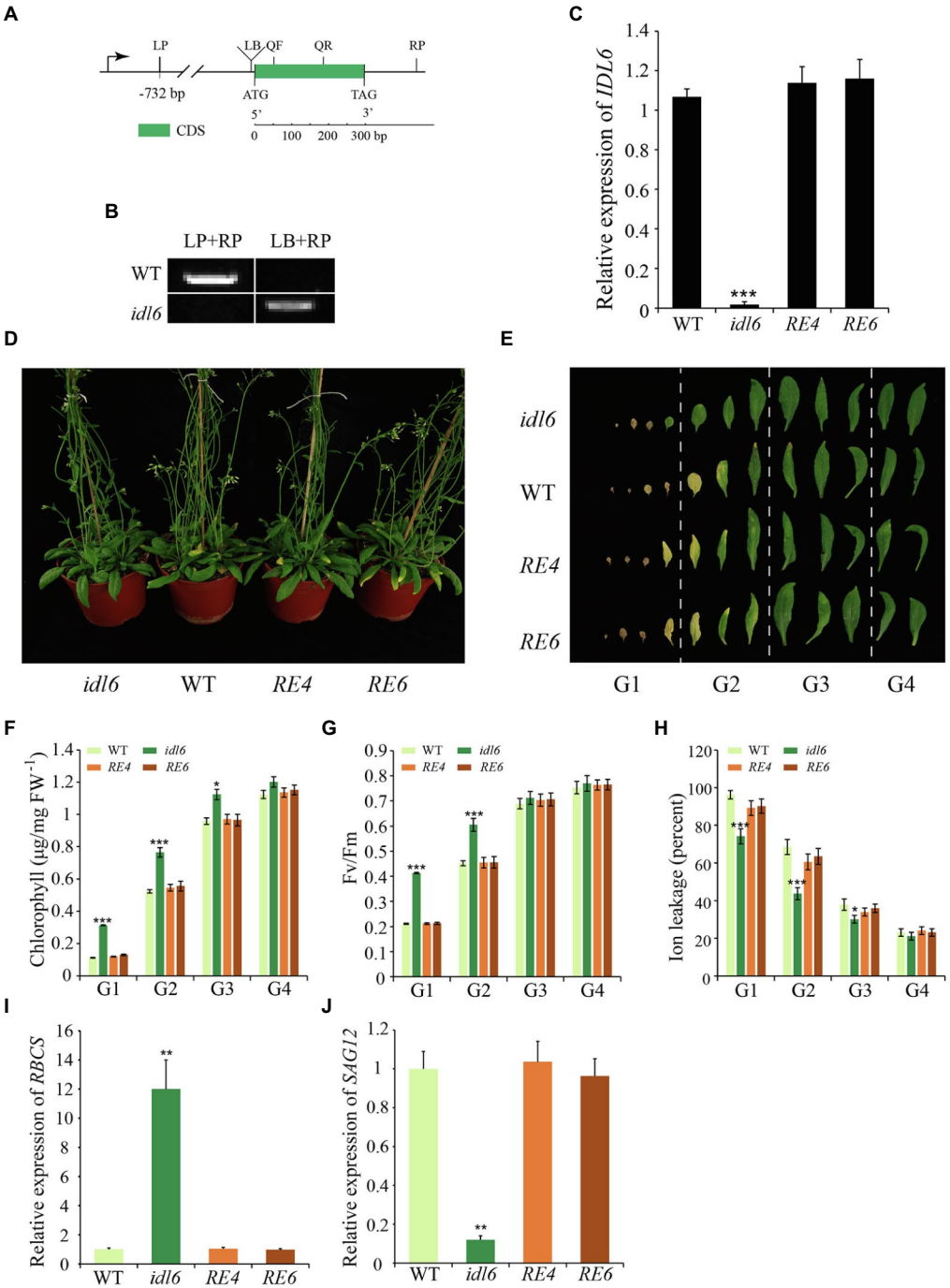

FIGURE 4 | Leaf senescence phenotypes of *IDL6* loss-of-function plants. **(A)** The gene structure of the *IDL6* gene and location of the T-DNA insertion. **(B)** Homozygous mutant plants were identified by PCR. LP: left primer, RP: right primer, LB: T-DNA left border primer. **(C)** Quantification of *IDL6* transcripts in wild-type, *idl6* mutant and *IDL6* rescue lines. **(D)** Leaf senescence phenotypes of 8-week-old wild-type, *idl6* mutant and *IDL6* rescue lines (*RE4* and *RE6*). **(E)** Phenotypes of 12 detached rosette leaves from wild-type, *idl6* mutant and *IDL6* rescue lines. Quantification of chlorophyll contents **(F)**, photosynthetic rates **(G)** and ion leakage **(H)** in leaves from each group. The relative expression of *RBCS* **(I)** and *SAG12* **(J)** of group 2 leaves from each line. The data are means ± SD of three biological repeats. Significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) compared with the wild type in each group were determined by Student's *t*-test.

plants (WT, *idl6*, *OE1*, *OE4*, *RE4*, and *RE6*), placed on moistened filter papers and incubated under darkness to induce senescence. Three days later, the chlorophyll levels of all leaves from the *IDL6 OE* lines were significantly reduced, while relatively less chlorophyll breakdown occurred in the leaves of *idl6* mutant plants. After 5 days of dark treatment,

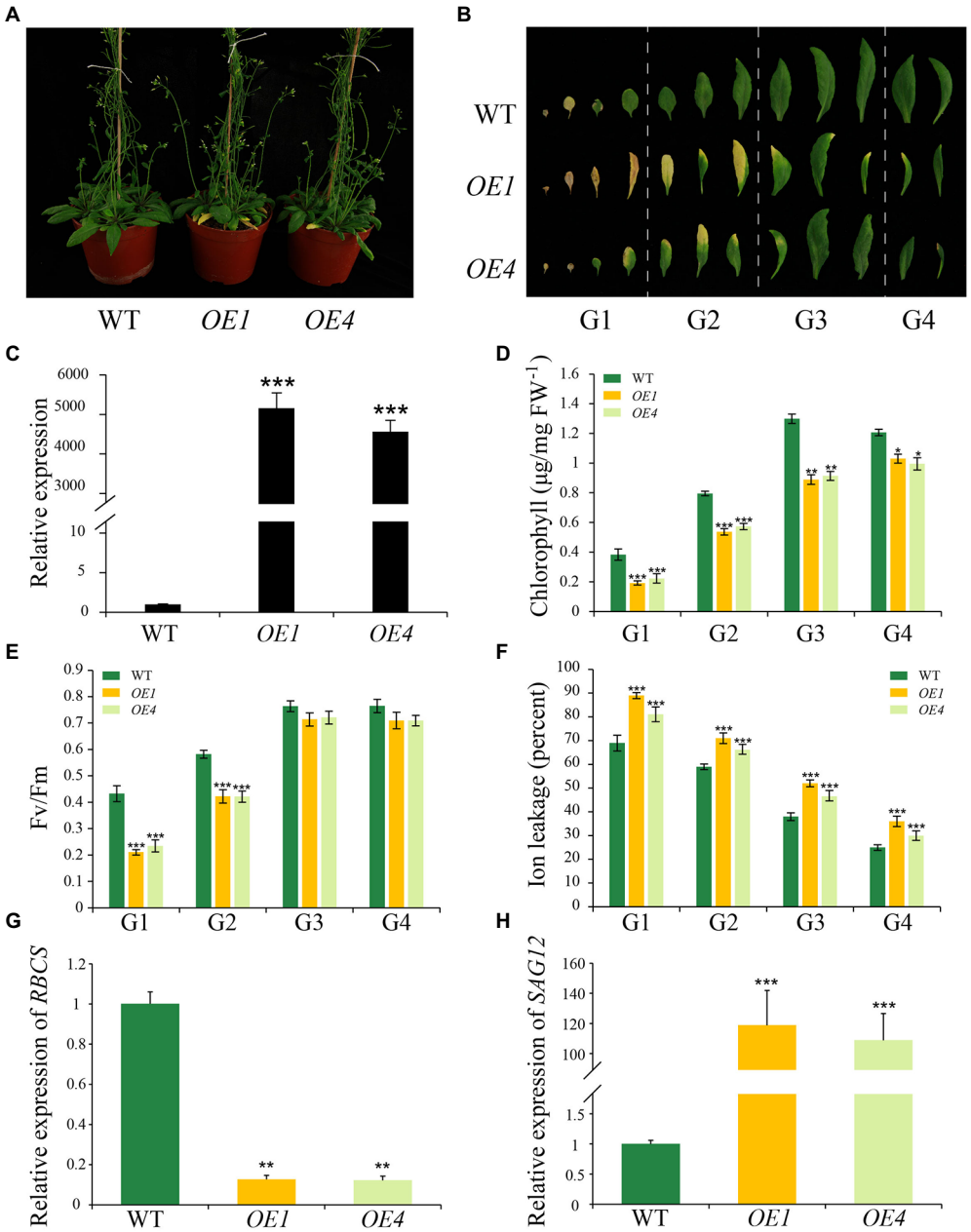

FIGURE 5 | Leaf senescence phenotypes of *IDL6* overexpression plants. **(A)** Leaf senescence phenotypes of 6-week-old wild-type and *IDL6* overexpression lines (*OE1* and *OE4*). **(B)** Phenotypes of 12 detached rosette leaves from wild-type and *IDL6* overexpression lines. **(C)** Relative expression of the *IDL6* gene in wild-type and *IDL6* overexpression lines. Quantification of chlorophyll contents **(D)**, photosynthetic rates **(E)** and ion leakage **(F)** in leaves from 4 groups of rosette leaves. The relative expression of *RBCS* **(G)** and *SAG12* **(H)** of group 2 leaves from each line. The data are means ± SD of three biological repeats. Significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) compared with the wild type in each group were determined by Student's *t*-test.

the early senescence phenotype became more obvious on leaves from *IDL6 OE* lines (**Figures 8A,B**). Specifically, the chlorophyll contents in *IDL6 OE* lines were ~2.94, ~3.13, and 2.94 times lower than that of the wild-type, *idl6* mutant and *IDL6 RE* lines, respectively. The changes in Fv/Fm also showed a similar trend (**Figures 8B,C**). Notably, overexpression of *IDL6* could also affect *RBCS* and *SAG12* expression in dark-induced senescence (**Supplementary Figure 4**). These results suggest that IDL6 is a positive regulator of dark-induced leaf senescence.

DISCUSSION

Being stationary in nature, plants have evolved a series of sophisticated mechanisms for responding to unpredictable

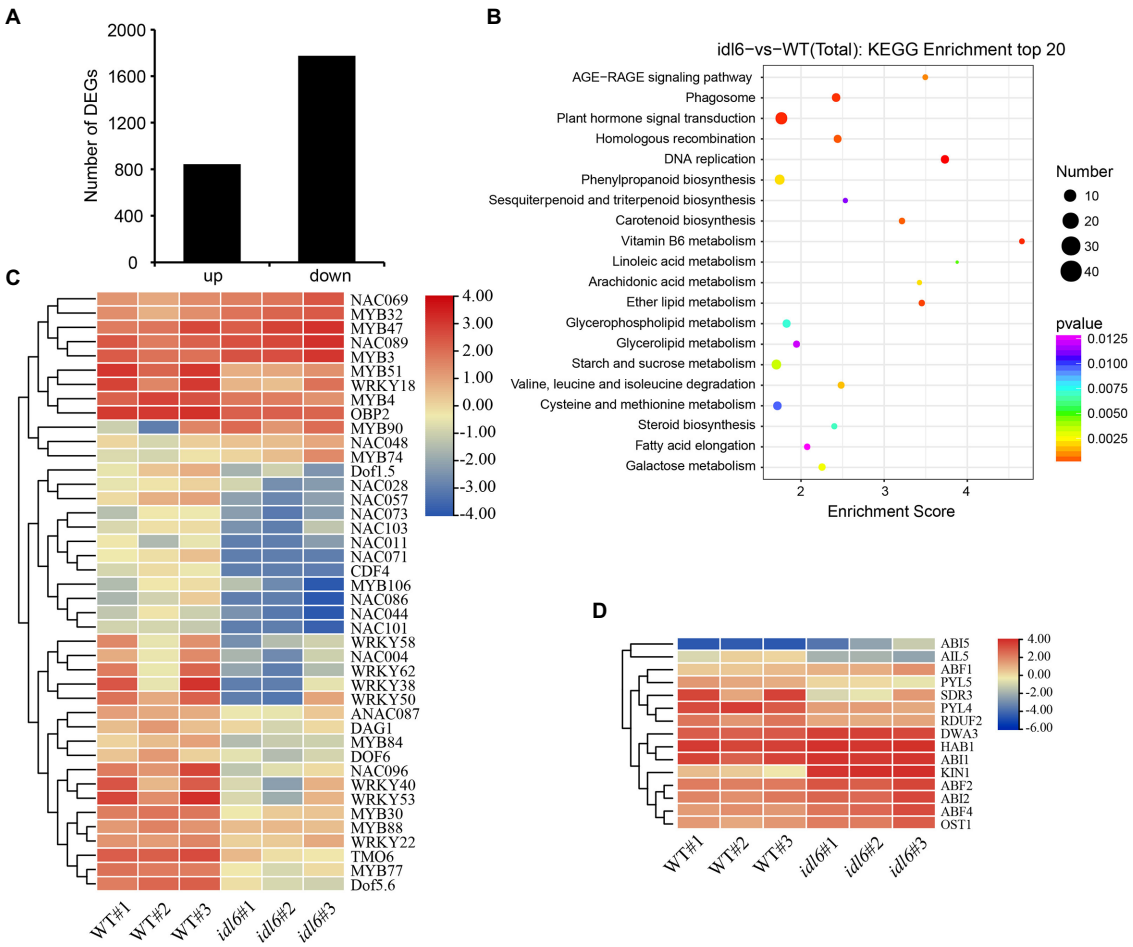

FIGURE 6 | Genome-wide transcriptome analysis of *idl6* mutant plants. **(A)** The number of up-regulated and down-regulated genes in rosette leaves of *idl6* mutant vs. Col-0. **(B)** KEGG bubble diagram of the DEGs in rosette leaves of *idl6* vs. Col-0. Bubble size represents numbers of DEGs, and bubble color represents *p*-values. The enrichment score represents the significance degree of DEGs in a certain signaling pathway. **(C)** Heatmap showing leaf senescence-related transcription factors in *idl6* mutant leaves compared with Col-0. The log2 fold change scale is indicated on the right side of the heatmap. **(D)** The heatmap of ABA-related genes in *idl6* mutant leaves compared to Col-0. The log2 fold change scale is indicated on the right side of the heatmap.

environmental stresses. Among them, cell-to-cell communication systems play a key role in growth and development. During the last decade, peptide ligands have emerged as vital mediators of cell-to-cell communications in plant growth, defense and stress responses in addition to the classical phytohormones (Matsubayashi and Sakagami, 2006). However, only one peptide, CLE14, has been functionally characterized and reported to be involved in leaf senescence (Zhang et al., 2021). In the current study, we found that *IDL6*, encoding a secreted peptide, was highly expressed in senescence leaves. Loss of *IDL6* function mutation delayed leaf senescence while *IDL6* overexpression and IDL6 peptide treatments caused precocious leaf senescence, supporting the role of IDL6 as a positive mediator of leaf senescence in *Arabidopsis*.

Comprising nine members (IDA and IDL1-8) in *Arabidopsis*, the IDL family members were detected to be expressed in floral organs, leaves, and roots. Among them, IDA has been shown to be important for flower abscission and lateral root emergence, while IDL6 and IDL7 have been reported to be negative regulators of genes associated with early responses to stresses (Vie et al., 2017). In a previous study, knockdown lines of *IDL6* showed increased resistance to *Pst* DC3000 in *Arabidopsis* (Wang et al., 2017). In this study, IDL6 peptides were found to function in promoting age-dependent leaf senescence and senescence induced by darkness, ABA and ethylene treatments. Cross-talks between plant senescence and stress responses have been well recognized in earlier studies (Guo and Gan, 2012; Guo et al., 2021). IDL6 could be induced rapidly by various biotic and abiotic stresses, such as cold, salt, UV, *P. syringae* (Vie et al., 2015). It might act as a signaling hub where different pathways interconnect with each other. Notably, peptides from the same family are often found to be involved in similar biological processes (Matsubayashi and Sakagami, 2006). It will be no surprise if some of the other IDL family peptides are also found to be involved in plant senescence.

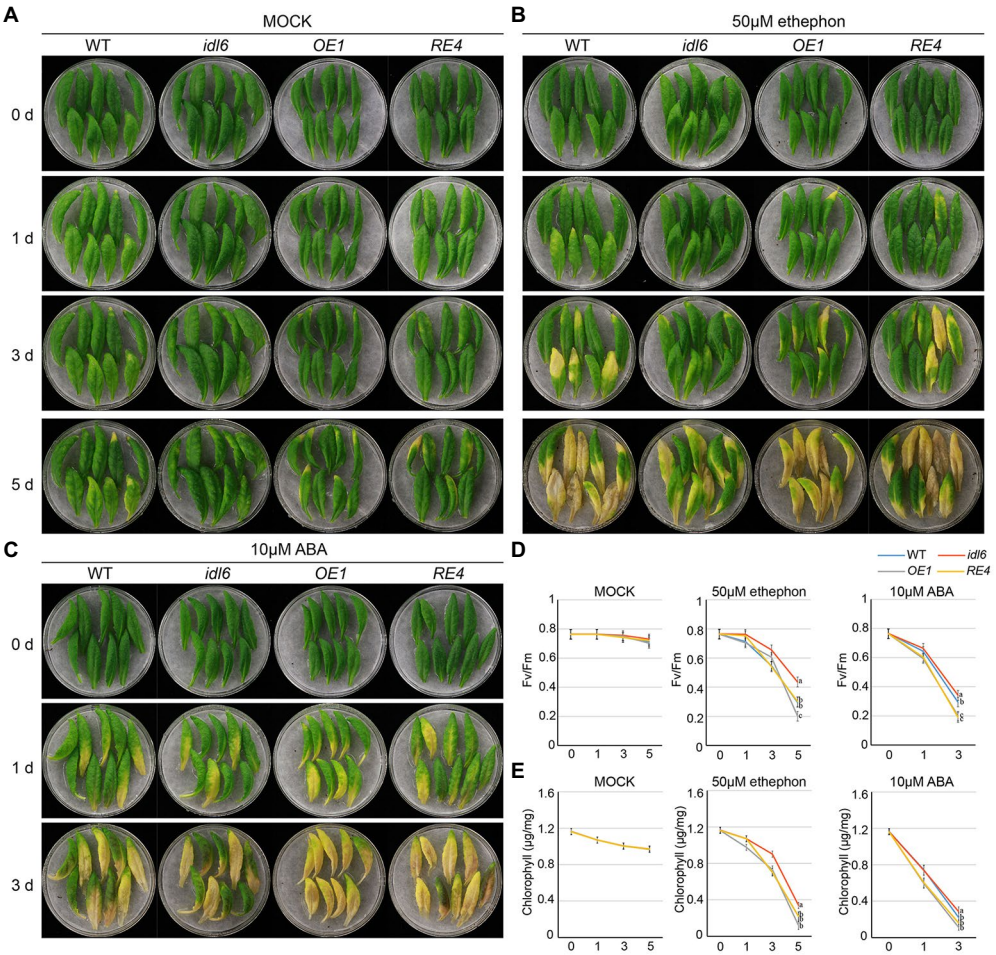

FIGURE 7 | Senescence induced by ABA and ethylene on detached leaves of *Arabidopsis*. The 7th, 8th and 9th leaves of 6-week-old wild type, *idl6* mutant, *IDL6* OE plants and *idl6* rescue plants were collected and treated by mock **(A)**, 50 μM ethephon **(B)** and 10 μM ABA **(C)**. The images of leaves were taken before and 1 day, 3 days, and 5 days after treatments. **(D)** The Fv/Fm and **(E)** chlorophyll concentrations in hormone treated and untreated leaves. For all conditions, statistically significant had been performed by two-way ANOVA analysis. All treatments were performed for three times.

Receptor-like kinases are indispensable sensors that contribute to intercellular communication, especially peptide ligand signaling (Diévart and Clark, 2003; Gish and Clark, 2011). At present, most receptors of peptides have been identified to be in the Leucine-rich repeat receptor-like kinases (LRR-RLK) family, such as RLK7, CEPR, BAM, and PEPR (Deyoung et al., 2006; Klauser et al., 2015; Zhou et al., 2022). As the receptors of the IDA peptide, HAE and HSL2 were reported to participate in flower abscission and lateral root emergence (Stenvik et al., 2008b; Kumpf et al., 2013). HAE and HSL2 also have been characterized as receptors of the IDL6 peptide in regulating plant disease resistance by activating cell wall synthesis genes (Wang et al., 2017). Peptide signals might be perceived by the same or different receptors when functioning in different biological processes (Zhang et al., 2021). Whether IDL6 functions through interacting with HAE and HSL2 in promoting leaf senescence remains to be elucidated.

The roles of phytohormones in leaf senescence have been well established (Guo and Gan, 2012). The transcriptome analysis in this study revealed multiple DEGs related to phytohormones in the *idl6* mutant (**Figures 6B,D**). Further study indicated that IDL6 functioned in leaf senescence induced by ABA and ethylene (**Figure 7**). How peptide signals including CLE14 and IDL6 interact with known senescence-regulating phytohormones and other senescence-regulating signals will be the next questions to be addressed in this field.

Leaf senescence as a complex and orderly controlled physiological process, requires hierarchical but also coordinated regulation by multiple transcription factors (Guo et al., 2004). The transcriptomic analyses of leaves from different developmental stages have identified numerous TFs differentially expressed during leaf senescence and more and more studies had characterized these TFs' functions in leaf senescence (Guo, 2013). In this study, we found that *WRKY53* and *CDF4* were

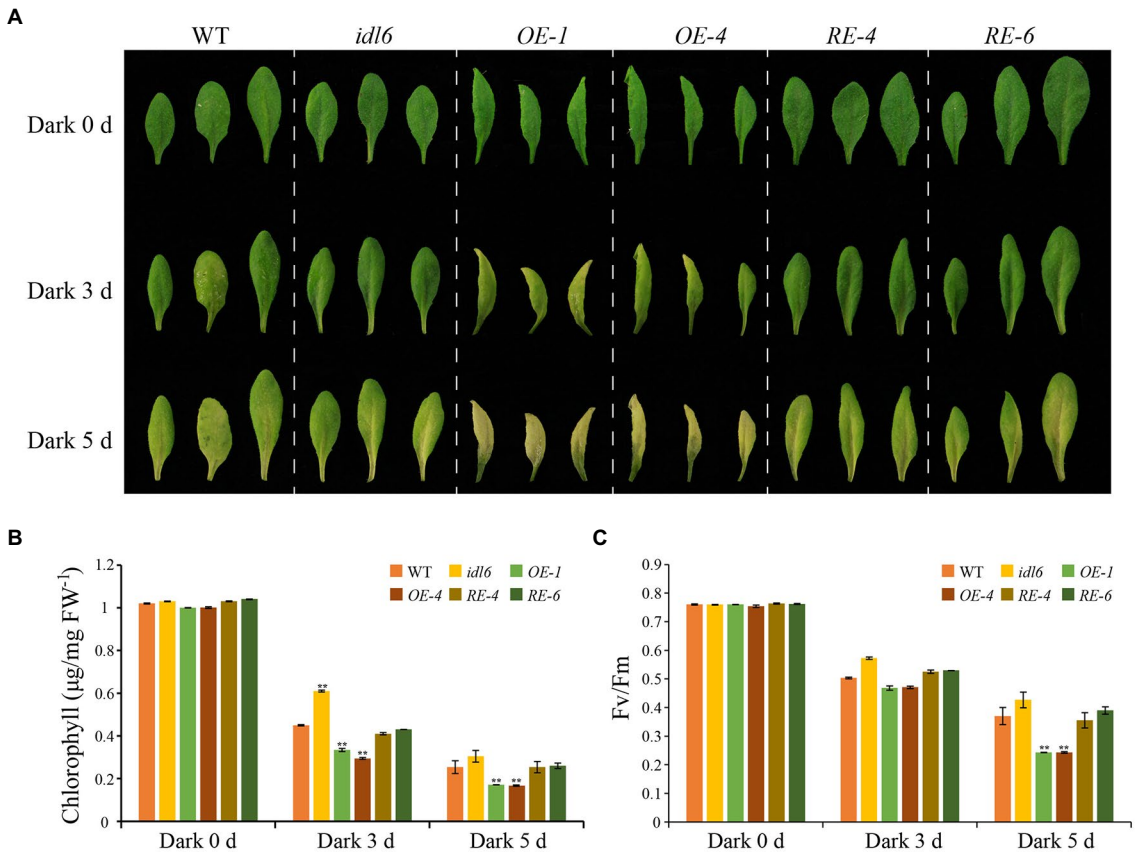

FIGURE 8 | Dark-induced senescence in detached leaves with altered *IDL6* expression. **(A)** The senescence phenotype s of the 5th, 6th, and 7th leaves from 4.5-week-old plants under dark treatment. Chlorophyll contents **(B)** and photosynthetic efficiency **(C)** of each leaf were measured after dark treatment for 0, 3 and 5 days. Error bars showed the SE ($n = 3$). Significant differences (**$p < 0.01$) compared with the wild type in each group were determined by Student's *t*-test. Three independent experiments were carried out with similar results.

down-regulated in the *idl6* mutant (**Figure 6C**), while they showed higher expression in overexpression *IDL6* gene line (**Supplemental Figure 3**). These results indicated that transcription factors WRKY53 and CDF4, both have been characterized as positive regulators of senescence (Miao et al., 2004; Xu et al., 2020), might function downstream of IDL6 in regulating leaf senescence. The specific regulatory mechanisms underlying the interactions between IDL6 and senescence-regulating transcription factors remain to be further studied.

CONCLUSION

In this study, we functionally characterized the IDL6 peptide, the encoding gene of which exhibited the highest expression level in naturally senescing leaves. Exogenous application of synthetic IDL6 EPIP peptides accelerated leaf senescence. Transgenic *Arabidopsis* plants with depleted or overexpressed *IDL6* had delayed or accelerated leaf senescence, respectively, indicating a positive role of IDL6 peptides in regulating leaf senescence. Furthermore, IDL6 peptides induced leaf senescence under darkness and hormonal treatments. Several senescence-associated transcription factors were significantly down-regulated in the *idl6* mutant, suggesting extensive cross talks between the IDL6 signal and known senescence-regulating pathways.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

CG and XL conducted the research and participated in drafting the manuscript. ZeZ, QW, ZhZ, LW, CL, ZD, YC, and TL assisted in data collection and analysis. YG conceived this research, designed the experiments, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.909378/full#supplementary-material

Supplementary Figure 1 | Sequence information of the IDL6 peptide.

Supplementary Figure 2 | Growth of wild-type, *idl6* mutant and a complementary line on 1/2 MS medium containing Kanamycin.

Supplementary Figure 3 | The relative expression of *WRKY53*, *CDF4*, *WRKY38*, and *WRKY62* in wild-type, *idl6* mutant, rescue and overexpression lines.

Supplementary Figure 4 | The expression levels of *RBCS* and *SAG12* in wild-type and overexpression plants under dark treatments.

Supplementary Figure 5 | The senescence phenotypes of detached leaves treated with 1 μM and 10 μM IDL6 peptides. **(A)** The detached leaves from wild type of *Arabidopsis* exhibited early senescence under IDL6 peptide treatments. Leaves were kept in a growth chamber at 23 °C with continuous light. Changes in chlorophyll content **(B)** and photosynthetic rate **(C)** in the treated leaves. Significant differences compared with the Mock were determined by Student's *t*-test (*$p < 0.05$, **$p < 0.01$).

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EDITED BY
Ying Miao,
Fujian Agriculture and Forestry University, China

REVIEWED BY
Masanori Izumi,
RIKEN, Japan
Yongfeng Guo,
Tobacco Research Institute (CAAS), China

*CORRESPONDENCE
Yasuhito Sakuraba
sakuraba0425@gmail.com

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Molecular basis of nitrogen starvation-induced leaf senescence

Yasuhito Sakuraba*

Plant Functional Biotechnology, Biotechnology Research Center, The University of Tokyo, Tokyo, Japan

Nitrogen (N), a macronutrient, is often a limiting factor in plant growth, development, and productivity. To adapt to N-deficient environments, plants have developed elaborate N starvation responses. Under N-deficient conditions, older leaves exhibit yellowing, owing to the degradation of proteins and chlorophyll pigments in chloroplasts and subsequent N remobilization from older leaves to younger leaves and developing organs to sustain plant growth and productivity. In recent years, numerous studies have been conducted on N starvation-induced leaf senescence as one of the representative plant responses to N deficiency, revealing that leaf senescence induced by N deficiency is highly complex and intricately regulated at different levels, including transcriptional, post-transcriptional, post-translational and metabolic levels, by multiple genes and proteins. This review summarizes the current knowledge of the molecular mechanisms associated with N starvation-induced leaf senescence.

KEYWORDS

leaf senescence, nitrogen (N), N starvation, N remobilization, transcriptional regulation

Introduction

Leaf senescence, the final phase of leaf development, is a highly controlled process accompanied by massive transcriptional and metabolic changes that destabilize intracellular organelles and macromolecules and lead to the translocation of nutrients into developing tissues and storage organs. In the past two decades, molecular mechanisms underlying the regulation of leaf senescence have been extensively studied (Woo et al., 2019; Guo et al., 2021; Zhang et al., 2021). The initiation of leaf senescence is tightly controlled by internal factors, such as the state of phytohormones, photosynthesis, sugars, and other metabolites (Sakuraba et al., 2012a; Jibran et al., 2013; Woo et al., 2019), and external stimuli such as high salinity, drought, pathogens, and light (Quirino et al., 1999; Gepstein and Glick, 2013; Zhang et al., 2012; Sakuraba, 2021a). In addition to these external stimuli, the deficiency of mineral nutrients in the soil is known to cause premature leaf yellowing.

Nitrogen (N) is a key mineral nutrient for plants and a major constituent of molecules essential for plant growth, such as nucleic acids, amino acids, and chlorophyll (Marschner, 1995). Thus, the availability of N is often a limiting factor for many aspects of plant growth and development. In the natural ecosystem and the field, plants frequently encounter N deficiency and thus exhibit N deficiency responses to efficiently acquire and use available N in the soil (Kiba and Krapp, 2016). N deficiency responses include the modification of root architecture (Gruber et al., 2013) and the expression of genes associated with high-affinity transport systems for nitrate and ammonium (Lezhneva et al., 2014; Kiba and Krapp, 2016) to promote the uptake of N sources. In addition, leaf yellowing due to the remobilization of N sources from older leaves to younger leaves and reproductive organs is also one of the representative N deficiency responses and is considerably important for plants to sustain growth and productivity. On the other hand, N starvation-induced leaf yellowing in young seedlings causes severe growth defects (Sakuraba et al., 2021b). Therefore, understanding the molecular mechanisms underlying N starvation-induced leaf senescence is critical for establishing sustainable agriculture under N-deficient conditions.

In recent years, the regulatory mechanisms of N starvation-induced leaf senescence have been widely uncovered at the transcriptional, post-transcriptional, post-translational, and metabolic levels. This review summarizes the results of studies conducted to date on N starvation-induced leaf senescence in the model dicot *Arabidopsis thaliana* and in agronomically important crops.

Metabolic changes in plants during N deficiency

Plants increase the capacity of N acquisition by enhancing root growth and upregulating the expression of genes encoding high-affinity nitrate and ammonium transporters under N deficiency stress (Kiba and Krapp, 2016). However, when these adaptations are not enough to provide a sufficient N supply, plants are forced to respond with further adaptive metabolic strategies that facilitate N remobilization to complete their life cycle.

The metabolome of Arabidopsis seedlings exposed to long-term N starvation showed dramatic changes (Krapp et al., 2011). Under N deficiency stress, the accumulation of nitrate and ammonium ions decreased rapidly. The total amino acid content of shoots gradually decreased during N starvation, while that of roots increased during the early phase of N starvation and then gradually returned to the level observed before the start of N starvation (Krapp et al., 2011). In shoots, the levels of N-rich amino acids such as glutamine (Gln), glutamate (Glu), asparagine (Asn), and aspartate (Asp) significantly decreased within a few days of N starvation; the accumulation of hydrophobic amino acids, such as leucine (Leu), isoleucine (Ile), and valine (Val), showed no significant change; and the levels of a few minor amino acids, such as lysine (Lys), arginine (Arg), and histidine (His), increased during long-term N starvation (Krapp et al., 2011). Since other N-containing compounds such as proteins and chlorophylls are synthesized from amino acids, the reduction in amino acid levels during long-term N starvation directly affects the accumulation of these compounds, leading to the promotion of leaf yellowing (Krapp et al., 2011; Balazadeh et al., 2014).

On the other hand, the content of soluble sugars such as sucrose, fructose, and galactose increased dramatically in Arabidopsis plants during N starvation (Krapp et al., 2011; Balazadeh et al., 2014). Several studies reported that sugars play an important role in the promotion of leaf senescence. Direct application of sucrose and glucose induced yellowing in *Xanthium pensylvanicum* leaf discs and Arabidopsis seedling leaves, respectively (Khudairi, 1970; Wingler et al., 2006). Moreover, genetic mutants and transgenic plants with altered sugar accumulation or sensing exhibited differences in the promotion of leaf senescence. Transgenic tomato plants overexpressing Arabidopsis *HEXOKINASE1* (*AtHXK1*) exhibited accelerated leaf senescence (Dai et al., 1999). On the other hand, an Arabidopsis deficient mutant of *MALTOSE EXCESS 1* (*MEX1*) exhibited a pale-green leaf phenotype and premature leaf senescence (Stettler et al., 2009). Thus, increased accumulation of soluble sugars in plants under N-deficient conditions may contribute to the promotion of leaf senescence.

N starvation also alters the accumulation of some organic acids. During N starvation, the levels of fumarate and succinate significantly increased, while those of aconitate and citrate decreased in the shoots of Arabidopsis seedlings (Krapp et al., 2011). While the accumulation of fumarate was shown to be closely associated with the accumulation of amino acids (Pracharoenwattana et al., 2010), the involvement of these organic acids in the promotion or inhibition of leaf senescence has not yet been investigated.

N deficiency induces the degradation of N-containing compounds and remobilization of N in older leaves

Under N deficiency stress, N is remobilized from senescing leaves to developing tissues, such as young leaves and other sink organs, in the form of nitrate, ammonium, urea, amino acids, and short peptides, leading to the promotion of leaf yellowing in older leaves. This N remobilization is accompanied by increased proteolysis activity in older leaves (Hörtensteiner and Feller, 2002). In addition, chlorophyll content, which is directly

associated with the amount of photosystem proteins, dramatically decreases under N-deficient conditions (Hanaoka et al., 2002). Section 3 summarizes the molecular mechanisms underlying the degradation of N-containing compounds and remobilization of N that occur during N deficiency stress.

Degradation of chloroplast proteins under N deficiency stress

In the mesophyll cells of C3 plants, approximately 80% of N is located in chloroplasts, mainly as a component of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; a stromal enzyme) and the light-harvesting complex (LHC; which contains chlorophyll pigments) (Peoples and Dalling, 1988; Makino et al., 2003). Previous studies showed that the accumulation of Rubisco dramatically decreased in the leaves of *Phaseolus vulgaris* (Crafts-Brandner et al., 1996) and Arabidopsis plants (Izumi et al., 2010) under N-deficient conditions. Rubisco and photosystem proteins are believed to be degraded under N deficiency stress through multiple proteolytic pathways, one of which is mediated by chloroplast proteases. Several studies reported the significance of chloroplast proteases in the degradation of photosystem proteins. For example, FtsH and DegP proteases are involved in the degradation of the damaged D1 protein (Lindahl et al., 2000; Haussühl et al., 2001), a core subunit of photosystem II (PSII). FtsH is also involved in the degradation of the Lhcb2 protein (Zelisko et al., 2005). Additionally, the chloroplast-localized aspartic protease CND41 was shown to mediate the degradation of Rubisco in tobacco leaves under N-deficient conditions (Kato et al., 2004). Although direct evidence is lacking, Rubisco is also speculated to be degraded by other stromal proteases, such as Clp (Figure 1). Indeed, Clp was shown to be involved in the degradation of Rubisco in the chloroplast of the green alga *Chlamydomonas reinhardtii* (Majeran et al., 2019).

Guiboileau and coworkers indicated the significance of autophagy in N remobilization under N deficiency stress. Tracer experiments using ^{15}N-labeled nitrate showed that N remobilization into seeds was reduced in autophagy mutants under N deficiency stress (Guiboileau et al., 2012). Furthermore,

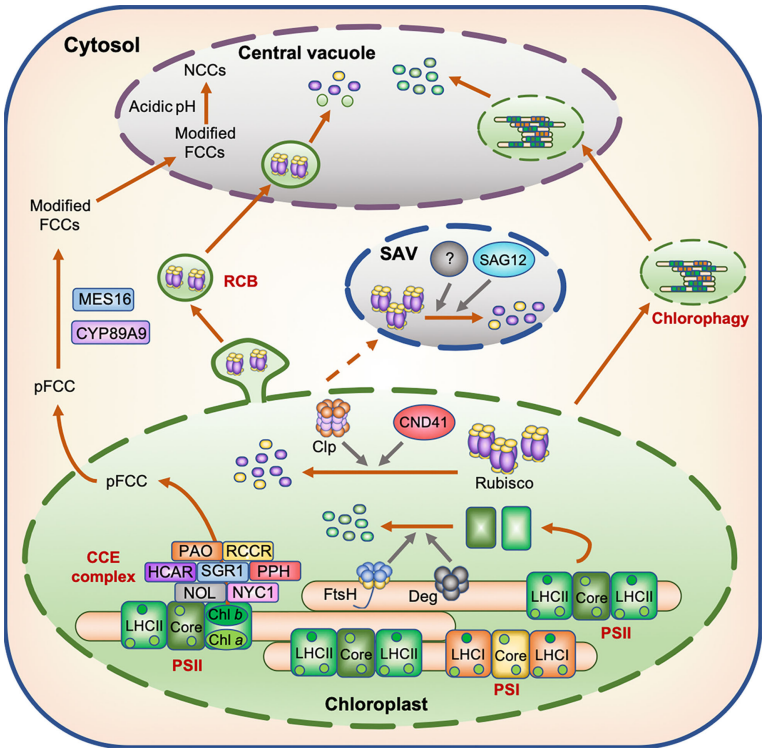

FIGURE 1
Model displaying the degradation of N-containing compounds in the chloroplast during leaf senescence. During leaf senescence, chloroplast proteins including Rubisco and photosystem subunits are believed to be degraded through several proteolytic processes mediated by proteases localized in the chloroplasts, senescence-associated vacuoles (SAVs), and central vacuoles as well as by Rubisco-containing bodies (RCBs) and chlorophagy. The degradation of chlorophyll molecules occurs in two distinct phases; the first phase is associated with the degradation of chlorophyll molecules by the chloroplast-localized chlorophyll catabolic enzyme (CCE) complex, while the second phase is associated with the translocation of colorless chlorophyll catabolites fluorescent chlorophyll catabolite (FCC) from chloroplasts to the central vacuole.

the N content of rosette leaves was significantly higher in autophagy mutants than in the wild type (WT) (Guiboileau et al., 2013). Although the number of Rubisco-containing autophagosomes, called Rubisco-containing bodies (RCBs), decreased during the period of N deficiency (Izumi et al., 2010), a certain amount of Rubisco proteins is speculated to be translocated into the central vacuole through the autophagy pathway, and then degraded (Figure 1). In Arabidopsis plants subjected to dark-induced senescence, chlorophyll fluorescence was detected in the central vacuole of leaf cells (Wada et al., 2009), indicating that the macroautophagy system also functions in the transport of chlorophyll–apoprotein complexes and other thylakoid proteins from chloroplasts to the central vacuole during dark-induced leaf senescence and probably under other senescence-inducing conditions including N deficiency. Chlorophagy, in which whole chloroplasts are transported to the central vacuole (Nakamura and Izumi, 2018), may also be involved in the transport of thylakoid proteins to the central vacuole during leaf senescence (Figure 1).

While autophagy is certainly involved in N remobilization, its molecular mechanism remains to be elucidated. In previous studies, Arabidopsis autophagy mutants exhibited early leaf yellowing under N-deficient conditions (Thompson et al., 2005; Guiboileau et al., 2012) and during dark-induced leaf senescence (Thompson et al., 2005), and exhibited leaf necrosis under abiotic stresses such as high salinity and drought (Liu et al., 2009). Under these stress conditions, however, chlorophyll degradation should be impaired when autophagy operates properly, since autophagy is involved in the degradation of chloroplast proteins. On the other hand, the leaves of Arabidopsis autophagy mutants exhibited delayed leaf yellowing under mild abiotic stress conditions (Sakuraba et al., 2014a). This difference in the progression of chlorosis (or necrosis) of autophagy mutant leaves between severe and mild stress conditions may reflect the significance of autophagy in adapting to severe stress. It is possible that autophagy mutants cannot adapt to severe stress, since they cannot properly maintain their proteome balance under extremely unfavorable conditions and thus exhibit accelerated leaf yellowing and/or leaf necrosis. Investigation of the phenotype of autophagy mutants under different N concentrations will provide important insights into autophagy-mediated N remobilization that occurs under N deficiency stress.

During leaf senescence, senescence-associated vacuoles (SAVs), which show greater lytic activity than the central vacuole, are formed in the peripheral cytoplasm of mesophyll cells (Otegui et al., 2005). SAVs contain stromal proteins such as Rubisco and glutamine synthetase (GS) but do not contain thylakoid proteins such as D1, LHC of PSII (LHCII), and cytochrome *c* (Cyt *c*) (Martínez et al., 2008), indicating that SAVs are involved in the degradation of stromal proteins, but not thylakoid proteins during senescence. In wheat (*Triticum aestivum* L.), the activity of several vacuolar cysteine proteases increased in senescing leaves (Martínez et al., 2007). *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*), which encodes a vacuolar cysteine protease, is one of the most widely used senescence marker genes. The expression of *SAG12* is strongly induced during leaf senescence (Lohman et al., 1994), and the encoded protein localizes to SAVs (Otegui et al., 2005). Therefore, SAG12 is thought to participate in the degradation of Rubisco proteins in SAVs (Figure 1). However, in the *sag12* knockout mutants of Arabidopsis, the degradation of Rubisco proteins was not affected under both high and low N conditions, whereas the activity of aspartic protease was greatly enhanced (James et al., 2018). These results suggest that some aspartic proteases compensate for the effect of *sag12* mutation on the degradation of Rubisco proteins. Thus, functional characterization of aspartic proteases in SAVs is necessary for further understanding the proteolytic process of stromal proteins in SAVs.

Degradation of chlorophyll pigments under N-deficient conditions

Under N deficiency stress, the timing of chlorophyll pigment degradation is consistent with that of photosystem protein degradation, and is accompanied by the loss of green color. The degradation of chlorophyll pigments occurs in two distinct phases. The first phase is associated with the degradation of chlorophyll pigments and their intermediates in the chloroplast, while the second phase involves the translocation of colorless chlorophyll catabolites from the chloroplast to the vacuole (Kuai et al., 2018). In the first phase, the degradation of chlorophyll molecules is catalyzed by at least seven enzymes. This catabolic process starts with the conversion of chlorophyll *b* to 7-hydroxymethyl chlorophyll *a* by two chlorophyll *b* reductase isoforms, NON-YELLOW COLORING 1 (NYC1) and NYC1-LIKE (NOL) (Kusaba et al., 2007; Horie et al., 2009), and is followed by the conversion of 7-hydroxymethyl chlorophyll *a* to chlorophyll *a* by 7-HYDROXYMETHYL CHLOROPHYLL *a* REDUCTASE (HCAR) (Meguro et al., 2011). The dechelation of magnesium from chlorophyll *a* is catalyzed by a magnesium-dechelatase, NON YELLOWING 1 (NYE1)/STAY-GREEN1 (SGR1) (Shimoda et al., 2016), and the product of this reaction (pheophytin *a*) is then dephytylated by PHEOPHYTINASE (PPH) to form pheophorbide *a* (Schelbert et al., 2009). Subsequently, the chlorin macrocycle of pheophorbide *a* is oxygenolytically opened by PHEOPHORBIDE *a* OXYGENASE (PAO) (Pruzinská et al., 2003) to form red chlorophyll catabolite (RCC), which is then reduced to a non-phototoxic chlorophyll catabolite, primary fluorescent chlorophyll catabolite (pFCC), by RCC REDUCTASE (RCCR) (Pruzinská et al., 2007). These seven chlorophyll catabolic enzymes physically interact with each other and with LHCII (Sakuraba et al., 2012b, Sakuraba et al., 2013), indicating that these chlorophyll catabolic enzymes form a multi-

protein, and potentially highly dynamic, complex for cellular detoxification during leaf senescence (Figure 1). During dark-induced and developmental leaf senescence, the expression levels of genes encoding some of the chlorophyll catabolic enzymes increase rapidly (Schelbert et al., 2009). In Arabidopsis, the expression levels of *NYC1*, *HCAR*, *NYE1/SGR1*, *PPH*, and *PAO* increased significantly under N deficiency stress (Figure 2), which implies that these enzymes are involved in chlorophyll degradation during N starvation-induced leaf senescence.

Amino acid metabolism during N deficiency-induced N remobilization

Gln and Asn residues are the major carriers of N in the phloem sap of higher plants (Hayashi and Chino, 1990), and thus their synthesis in source organs is considerably important for N remobilization. The glutamine synthetase/glutamine-2-oxoglutarate aminotransferase (GS/GOGAT) cycle is considered to be the primary route of N assimilation in higher plants (Xu et al., 2012). In this cycle, GS catalyzes the synthesis of Gln from ammonium and glutamate (Glu) (Lam et al., 2003), while glutamate synthetase GOGAT catalyzes the transfer of the amide group of Gln to 2-oxoglutarate (2-OG) to synthesize two molecules of Glu (Suzuki and Knaff, 2005). On the other hand, asparagine synthetase (ASN) catalyzes the conversion from Glu to Asp to form Asn, and plays a vital role in N assimilation, remobilization, and allocation within the plant (Gaufichon et al., 2010).

Enzymes associated with the accumulation of Gln and Asn play important roles in the regulation of leaf senescence. The Arabidopsis genome harbors three *ASN* genes (*AtASN1–3*), among which *AtASN2* is the most highly expressed in the shoots (Gaufichon et al., 2010). The *atasn2* knockout mutants exhibit delayed leaf yellowing and relatively low *SAG12* expression compared with the WT during developmental senescence (Gaufichon et al., 2013). Similarly, in rice (*Oryza sativa* L.), knockout mutation of *OsASN1*, encoding one of the two ASN isoforms that plays a major role in Asn synthesis in roots (Ohashi et al., 2015), leads to the stay-green phenotype during developmental senescence (Lee et al., 2020). Delayed leaf yellowing observed in *asn* knockout mutants is probably caused by the decline in Asn synthesis, which likely leads to the stabilization of chlorophyll and other N-containing compounds in leaves. On the other hand, a rice knockout mutant of the gene encoding ferredoxin-dependent GOGAT (*OsFd-GOGAT*), which accumulates 10-fold more Gln than the WT, exhibited accelerated leaf yellowing during the reproductive phase, probably because of the promotion of N remobilization from old leaves to young leaves and other sink organs (Zeng et al., 2017).

The export of amino acids from older leaves is an important process for N remobilization under N deficiency stress. In poplar (*Populus trichocarpa*), the Gln content of senescing leaves

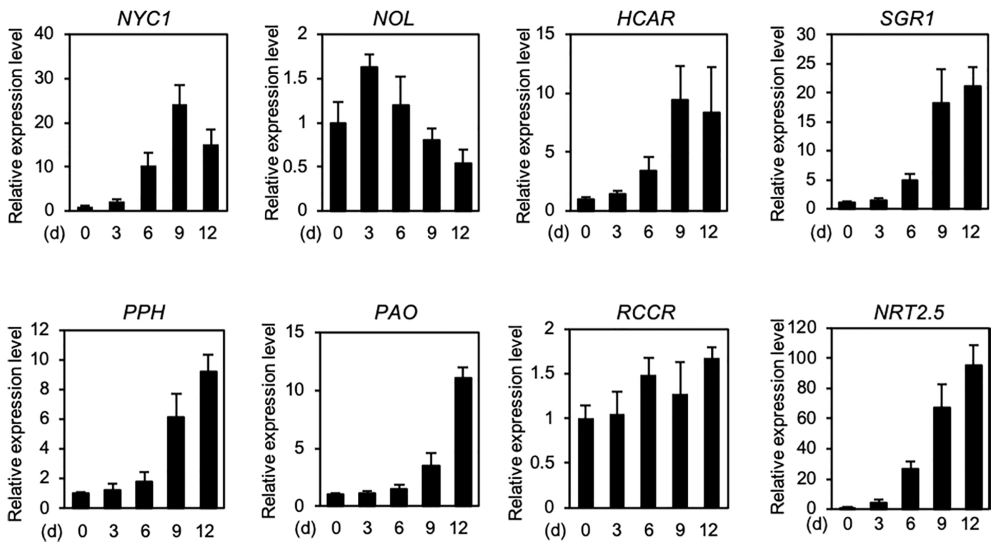

FIGURE 2
Expression profiles of seven genes encoding chlorophyll catabolic enzymes during N deficiency. Expression levels of seven chlorophyll catabolic enzyme-encoding genes *NYC1*, *NOL*, *HCAR*, *SGR1*, *PPH*, *PAO*, and *RCCR*, and a high-affinity nitrate transporter-encoding gene *NRT2.5* (positive control) in the shoots of Arabidopsis Col-0 (wild type) seedlings are shown. Plants were grown in plates containing half-strength Murashige and Skoog (1/2 MS)-agar medium for 7 days and then under N-deficient conditions (0.3 mM N) for the indicated time periods. Transcript levels of each gene were normalized against the transcript levels of *ACTIN2* (*ACT2*) and then against the value obtained from samples at time zero. Data represent mean ± standard deviation (SD) of four biological replicates.

dramatically increased, and a cationic amino acid transporter, Pt-CAT11, played an important role in the transfer of Gln during the senescence process (Couturier et al., 2010). In rice, overexpression lines of *OsAAP3* exhibited accelerated leaf yellowing phenotype, whereas RNA interference (RNAi) lines of *OsAAP3* exhibited delayed leaf yellowing (Wei et al., 2021). Higher plants possess a number of amino acid transporters (Yao et al., 2020); however, their functions in N remobilization during N deficiency stress remain unknown.

Roles of nitrate transporters in N remobilization

Recent studies in Arabidopsis showed that NRTs play a critical role in the regulation of N starvation-induced leaf senescence and N remobilization. The Arabidopsis genome possesses 53 and 7 genes encoding Nitrate Transporter 1 (NRT1)/Peptide Transporter (PTR) and NRT2 proteins, respectively (Tsay et al., 2007). NRT2 proteins function as high-affinity nitrate transporters, while most NRT1/PTR family proteins have been functionally characterized as low-affinity nitrate transporters (Krapp et al., 2014; Forde, 2000).

Among the NRT1/PTR family proteins, NRT1.1 is considered as a unique protein, since it acts as a dual affinity transporter that can facilitate nitrate uptake at concentrations ranging from micromolar to millimolar (Liu et al., 1999). Genome-wide association study (GWAS) of Arabidopsis accessions suggested a significant association between differences in N starvation-induced leaf yellowing and *NRT1.1* sequence diversity (Sakuraba et al., 2021b). Indeed, *nrt1.1* knockout mutant, *chl1-5* exhibited accelerated leaf yellowing, while transgenic *NRT1.1*-overexpressing (*NRT1.1*-OX) plants retained greenness under N-deficient conditions (Sakuraba et al., 2021b). Furthermore, grafted seedlings generated using *NRT1.1*-OX scion and WT (Col-0) rootstock exhibited delayed leaf yellowing under the N-deficient conditions; however, such a delayed leaf yellowing phenotype was not conserved when chimeras were generated by grafting WT (Col-0) on *NRT1.1*-OX rootstock (Sakuraba et al., 2021b), indicating that the enhanced expression of *NRT1.1* in aboveground plant parts negatively regulates N starvation-induced leaf yellowing.

Arabidopsis NRT1.5, which is also classified into the NRT1/PTR family, is involved in the nitrate loading of the xylem (Lin et al., 2008). The expression of *NRT1.5* is highly upregulated during leaf senescence (van der Graaff et al., 2006). Leaves of *nrt1.5* knockout (*nrt1.5*-KO) mutant plants turned yellow at a rate comparable with those of WT plants when grown under low N (i.e., low nitrate) conditions; however, leaves of the *nrt1.5*-KO mutant turned yellow much earlier than those of WT plants under low N conditions, when the only N source was ammonium or amino acids (Meng et al., 2016), indicating that accelerated leaf yellowing in *nrt1.5*-KO mutants is caused specifically by the nitrate starvation. Furthermore, the accelerated leaf yellowing phenotype of *nrt1.5*-KO mutants was diminished by supplementation with 10 mM foliar potassium (K). Additionally, K supply during nitrate starvation suppressed the expression of several genes associated with K acquisition, including *HIGH-AFFINITY* K^+ *TRANSPORTER 5* (*HAK5*), which encodes a major transporter that contributes to K uptake by roots (Gierth et al., 2005), and *RAP2.11*, which encodes a transcriptional regulator of *HAK5* (Kim et al., 2012), in *nrt1.5*-KO mutants (Meng et al., 2016). K supplementation also inhibited stress-induced yellowing of flag leaves in barley (*Hordeum vulgare* L.) (Hosseini et al., 2016). These findings suggest that NRT1.5 suppresses nitrate starvation-induced leaf senescence by modulating the K level.

Arabidopsis *NRT1.7*, which encodes a low-affinity nitrate transporter, is highly expressed in the phloem tissues of leaves, and its expression increases as leaves age (Fan et al., 2009). The *nrt1.7*-KO mutants highly retained nitrate in older leaves, and exhibited severe growth defects and premature leaf yellowing phenotype compared with WT plants when grown under N deficiency stress (Fan et al., 2009). Other Arabidopsis NRT1 family members, including NRT1.11 and NRT1.12, are also involved in nitrate remobilization from older to younger leaves. Both *NRT1.11* and *NRT1.12* are highly expressed in fully expanded rosette leaves, and the *nrt1.11 nrt1.12* double mutant exhibits lower nitrate content than the WT (Hsu and Tsay, 2013). However, the role of *NRT1.11* and *NRT1.12* in the response to N deficiency stress remains unknown. Considering the functions of NRT1 family proteins in the regulation of N starvation-induced leaf senescence, impaired nitrate remobilization in specific tissues may contribute to overall N deficiency, leading to the promotion of leaf senescence.

On the contrary, the involvement of NRT2 family proteins in the regulation of N deficiency-induced leaf senescence has not yet been elucidated. However, the expression levels of four Arabidopsis *NRT2* genes (*NRT2.1*, *NRT2.2*, *NRT2.4*, and *NRT2.5*) significantly increase during N deficiency (Lezhneva et al., 2014). While *NRT2.1*, *NRT2.2*, and *NRT2.4* are dominantly expressed in roots, *NRT2.5* is also expressed in shoots and is upregulated during N deficiency (Lezhneva et al., 2014). Thus, it is likely that some of the NRT2 family proteins also play important roles in the regulation of N starvation-induced leaf senescence.

Transcriptional regulatory network of N starvation-induced leaf senescence

To cope with N deficiency stress, plants increase the capacity of N acquisition by enhancing the expression of genes associated with high-affinity transport systems for nitrate and ammonium (Kiba and Krapp, 2016). When these adaptations are not enough

to provide a sufficient N supply, however, plants are forced to respond with further adaptive transcriptomic strategies for the remobilization of N to complete their life cycle.

In the last two decades, a number of leaf senescence-associated transcription factors have been identified and characterized in Arabidopsis and other plant species (Woo et al., 2019; Guo et al., 2021), which has greatly expanded our knowledge of the transcriptional regulatory network of leaf senescence. The functions of these senescence-associated transcription factors have been studied mostly during developmental progression-induced natural senescence or dark-induced leaf senescence (Guo and Gan, 2006; Kim et al., 2009; Kim et al., 2013; Sakuraba et al., 2014b). While dark-induced leaf senescence is known to be partially caused by the decline in N metabolism (Watanabe et al., 2013), the transcriptional regulatory network involved in this process remains unclear. Section 4 summarizes the recently reported transcriptomic changes that occur during N deficiency and the key regulatory modules involved in N starvation-induced leaf senescence.

Transcriptomic changes in plants during N deficiency

Changes of the transcriptome of Arabidopsis plants during N starvation have been investigated using several different experimental approaches. Scheible et al. (2004) used seedlings grown initially in N-replete liquid medium and then in N-limited liquid medium for several days for DNA microarray analysis (Scheible et al., 2004). Bi et al. (2007) used the leaves of 3-week-old Arabidopsis plants grown hydroponically under mild and severe N deficiency (1 and 0.3 mM N, respectively) for DNA microarray analysis (Bi et al., 2007). Balazadeh et al. (2014) used leaves collected from Arabidopsis plants initially grown under N-sufficient conditions for 19 days and then grown under N-free conditions for several additional days to perform DNA microarray analysis (Balazadeh et al., 2014). Although Krapp et al. (2011) used a similar approach for growing Arabidopsis plants as described above, these plants were grown under short-day (8 h light/16 h dark) photoperiod (Krapp et al., 2011).

In the transcriptome data obtained from these studies, several sets of genes were commonly up- or downregulated. For instance, several genes associated with anthocyanin biosynthesis, including *CHALCONE SYNTHASE* (*CHS*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*), and *PAP2*, were significantly upregulated in the leaves of plants exposed to N starvation. On the other hand, genes associated with cell wall organization, including *EXPANSIN A1* (*EXPA1*) and *EXPA8*, as well as those associated with photosynthesis and chlorophyll synthesis, including *HEMA1* and *HEME2*, were downregulated in shoots under N starvation (Scheible et al., 2004; Krapp et al., 2011). These observations were expected, since anthocyanin accumulation in shoots increases while shoot growth rate and photosynthetic activity decline under N starvation.

In addition, a number of senescence-associated genes were differentially regulated under N starvation. In the DNA microarray analysis performed by Balazadeh et al. (2014), more than half of the N starvation-induced genes were upregulated during developmental senescence upregulated genes, including *SAG12*, *SAG13*, and *ANAC029/NAC-LIKE, ACTIVATED BY AP3/PI* (*NAP*) (Buchanan-Wollaston et al., 2005; Breeze et al., 2011; Balazadeh et al., 2014). Upregulation of *NAP* under N starvation has also been reported in other transcriptome analyses (Scheible et al., 2004; Bi et al., 2007; Krapp et al., 2011; Balazadeh et al., 2014). NAP is classified into NO APICAL MERISTEM/ATAF1,2/CUP-SHAPED COTYLEDON (NAC) transcription factor family, and acts as an enhancer of developmental senescence and dark-induced leaf senescence by directly upregulating the expression of senescence-associated genes, including *SAG113* and *ABSCISIC ALDEHYDE OXIDASE 3* (*AAO3*) (Guo and Gan, 2006; Lei et al., 2020). In addition, the senescence-inducible gene *DUF581* was upregulated under N starvation in all transcriptome analyses described above (Krapp et al., 2011; Balazadeh et al., 2014).

Transcriptomic changes under N starvation-induced leaf senescence in the oilseed rape (*Brassica napus*) have been investigated using the cultivars that exhibit different responses to N starvation: NPZ-1 and Apex cultivars exhibit stay-green, while NPZ-2 and Capitol exhibit accelerated leaf yellowing under N deficient conditions (Schulte auf'm Erley et al., 2007; Koeslin-Findeklee et al., 2015a). As in the case in Arabidopsis, N deficiency in the leaves of oilseed rape also induced several senescence-associated genes, including *NAP* and *SGR1*, and some of these senescence-associated genes were highly expressed in the early senescing NPZ-2 and Capitol cultivars than in the stay-green NPZ-1 and Apex cultivars (Koeslin-Findeklee et al., 2015a). Moreover, biologically inactive cytokinins highly accumulated in the early senescing NPZ-1 and Apex cultivars, probably due to the altered expression of genes involved in the cytokinin homeostasis, including *CYTOKININ OXIDASE/DEHYDROGENASE2* (*CKX2*) (Koeslin-Findeklee et al., 2015b). Since the cytokinins are senescence-delaying phytohormones (Gan and Amasino, 1995), the homeostasis of biologically active cytokinins may be one of the predominant factors for the differences in N starvation-induced leaf senescence among cultivars of oilseed rape.

Roles of NAC transcription factors in the promotion of N deficiency-induced leaf senescence

The NAC family is one of the plant-specific transcription factor families (Riechmann et al., 2000). To date, a number of NAC transcription factors have been identified in Arabidopsis

and other plant species as key regulators of leaf senescence (Sakuraba et al., 2015; Kim et al., 2016; Pimenta et al., 2016; Sakuraba et al., 2020). Among the senescence-associated NAC transcription factors in Arabidopsis, the functions of ORESARA1 (ORE1)/ANAC092 have been widely studied. A number of studies revealed that ORE1 acts as a central regulator of both developmental senescence and dark-induced leaf senescence. Additionally, the regulatory cascades for the induction of *ORE1* (Kim et al., 2009; Sakuraba et al., 2014b; Kim et al., 2018; Yu et al., 2021) and its downstream target genes (Matallana-Ramirez et al., 2013; Qiu et al., 2015) have been identified. A recent study showed that ORE1 also acts as a key regulator of N starvation-induced leaf senescence. On N-deficient growth medium, leaves of the *ore1* knockout mutant turned yellow much faster than those of the WT, while the leaves of *ORE1* overexpressors (*ORE1*-OX) retained their green color (Park et al., 2018). Under N deficiency, the transcript level of *ORE1* was elevated 15–20-fold. In addition, the mRNA levels of *PHOSPHATE 2* (*PHO2*), encoding a ubiquitin-conjugating E2 enzyme (Bari et al., 2006), and *NITROGEN LIMITATION ADAPTATION* (*NLA*), encoding an E3 ubiquitin ligase that acts together with PHO2 (Park et al., 2014), were also elevated during N deficiency (Park et al., 2018). ORE1 interacts with NLA in the nucleus and then destabilized through polyubiquitination by the NLA–PHO2 module (Park et al., 2018). On the other hand, ubiquitin-specific protease 12 (UBP12) and UBP13, which act redundantly in the de-ubiquitination of target proteins (Derkacheva et al., 2016; Jeong et al., 2017), remove the ubiquitin moieties from the polyubiquitinated ORE1 protein, restoring its stable state. Thus, these two deubiquitinases counteract the effect of the NLA–PHO2 module during ORE1-mediated N starvation-induced leaf senescence (Figure 3).

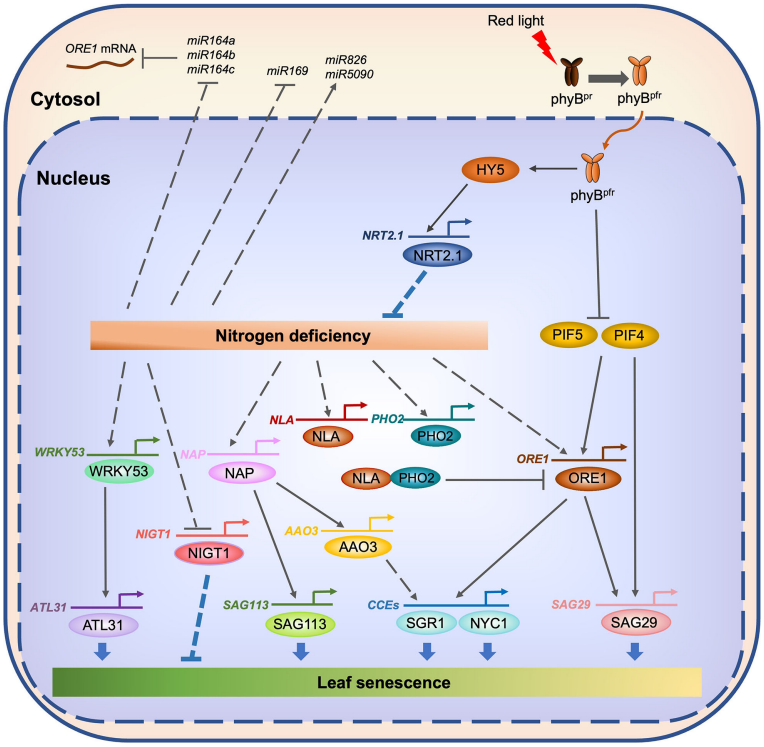

FIGURE 3
Transcriptional regulatory network of N starvation-induced leaf senescence in Arabidopsis. Under N-deficient conditions, the expression of several senescence-associated genes, including *WRKY53*, *NAP*, and *ORE1*, is enhanced. On the other hand, N deficiency downregulates the expression of *miR164*, which destabilizes *ORE1* mRNA, leading to a further increase of the accumulation of ORE1 protein. WRKY53 directly enhances the expression of *ATL31*, a key regulator of high C/low N-induced leaf senescence. NAP directly enhances the expression of *SAG113* and *AAO3*. ORE1 directly enhances the expression of *SAG29* and genes encoding chlorophyll catabolic enzymes (CCEs) including *SGR1* and *NYC1*. On the other hand, the expression of two genes associated with N deficiency responses, *NLA* and *PHO2*, is also enhanced. The NLA and PHO2 proteins promote the ubiquitination of ORE1, which leads to the degradation of ORE1, thus allowing the maintenance of a proper ORE1 protein level during N starvation-induced leaf senescence. phyB-mediated red light signaling may involve the suppression of N starvation-induced leaf senescence. Under the red light, the active Pfr form of phyB moves from cytosol to the nucleus. Under the downstream of phyB, HY5 directly activates the expression of genes associated with N acquisition, including *NRT2.1*. On the other hand, phyB promotes the proteasomal degradation of PIF4 and PIF5, which directly activate the expression of several senescence associated genes, including *ORE1* and *SAG29*. Solid lines indicate direct regulation, while dotted lines indicate indirect regulation.

In apple (*Malus domestica*), MdNAC4 participates in N starvation-induced leaf senescence. When grown under N-deficient conditions, the leaves of *MdNAC4*-overexpressing plants turned yellow faster than those of the WT, whereas the leaves of *MdNAC4*-antisense plants retained greenness (Wen et al., 2022). MdNAC4 was shown to directly enhance the expression of two genes encoding chlorophyll catabolic enzymes, *MdNYC1* and *MdPAO* (Wen et al., 2022). Additionally, MdNAC4 physically interacts with PSEUDO-RESPONSE REGULATOR2 (MdAPRR2), which enhances the expression of genes encoding chlorophyll biosynthesis enzymes, including MdHEMA, MdCHLI, and MdCHLM, to inhibit the activity of MdAPRR2 (Wen et al., 2022). Thus, the interaction between MdNAC4 and MdAPRR2 appears to be a balancing mechanism for regulating N starvation-induced leaf senescence in apple.

Roles of WRKY53 and ATL31 in the regulation of high carbon (C)/low N-induced leaf senescence

The carbon (C) status of plants affects N deficiency-induced leaf senescence. The rosette leaves of Arabidopsis WT (Col-0) plants turned yellow under high C and low N conditions (780 ppm CO_2 and 0.3 mM N) but not under low C and low N conditions (280 ppm CO_2 and 0.3 mM N), even at the same growth stage (Aoyama et al., 2014), indicating that the C status of plants is one of the key determinants of N starvation-induced leaf senescence. ARABIDOPSIS TOXICOS EN LEVADURA 31 (ATL31), a RING-type ubiquitin ligase, regulates the balance between C and N availability in Arabidopsis (Sato et al., 2009). Under high C/low N conditions, the leaves of *atl31*-KO plants turned yellow faster than those of WT plants, while the leaves of *ATL31*-OX plants retained greenness much longer (Aoyama et al., 2014), indicating that ATL31 acts as a negative regulator of high C/low N-induced leaf senescence. The transcript level of *ATL31* significantly increases under high C/low N conditions, similar to the expression pattern of the Arabidopsis *WRKY53* gene, which encodes a senescence-associated WRKY transcription factor (Miao et al., 2004; Miao and Zentgraf, 2007). WRKY53 was shown to activate the promoter of *ATL31* (Aoyama et al., 2014), indicating that WRKY53 acts as an enhancer for the induction of *ATL31* under high C/low N conditions (Figure 3). WRKY53 is one of the most widely studied senescence-associated transcription factors in Arabidopsis, and several key factors in the regulation of *WRKY53* expression and protein activity have been identified and characterized (Miao et al., 2013; Zentgraf and Doll, 2019; Doll et al., 2020). Additionally, the downstream targets of WRKY53 have been identified (Miao et al., 2004). While Aoyama et al. (2014) showed that WRKY53 enhances the expression of *ATL31*, it is still not clear how WRKY53 affects the promotion of N starvation-induced leaf senescence. Investigation of the effects of knockout mutation and overexpression of *WRKY53* on the promotion of N deficiency-induced leaf senescence is necessary for understanding the significance of the WRKY53–ATL31 regulatory module in N starvation-induced leaf senescence.

Possible involvement of phytochrome B-mediated red light signal in the regulation of N starvation-induced leaf senescence

While red light has long been considered to delay leaf senescence (Pfeiffer and Kleudgen, 1980; Tucker, 1981), the molecular mechanisms underlying red light signaling-mediated regulation of leaf senescence were partially revealed only in the last decade, especially in the model plant Arabidopsis. Among five phytochromes in Arabidopsis, namely phyA, phyB, phyC, phyD, and phyE (Sharrock and Clack, 2002), phyA plays a major role in the far-red light response (Reed et al., 1994). On the other hand, phyB–phyE are involved in the red/far-red low-fluence response *via* the reversible transition between the red-light-absorbing biologically inactive Pr form and the far-red-light-absorbing biological active Pfr form (Li et al., 2011). Among five Arabidopsis phytochromes, phyB is involved in the regulation of dark-induced leaf senescence; two *phyB* knockout mutants, namely *phyB-9* and *phyB-10*, exhibited accelerated leaf yellowing, while *phyB* overexpressors highly retained greenness after the dark incubation (Sakuraba et al., 2014b). PHYTOCHROME INTERACTING FACTOR4 (PIF4) and PIF5 act downstream of phyB, and promote both age-dependent and dark-induced leaf senescence by directly enhancing the expression of senescence-associated genes, including *ORE1*, *ETHYLENE INSENSITIVE 3* (*EIN3*), *ABA INSENSITIVE 5* (*ABI5*), *ENHANCED EM LEVEL* (*EEL*), *SGR1*, and *SAG29* (Sakuraba et al., 2014b; Song et al., 2014; Zhang et al., 2015; Sakuraba et al., 2017). In rice, phyB-mediated red light signaling is also involved in the promotion of dark-induced leaf senescence; the *osphyB* T-DNA insertion knockout mutant exhibited accelerated leaf yellowing during dark-induced leaf senescence (Piao et al., 2015). In addition, RNAi of *PIF4* in tomato (*Solanum lycopersicum* L.) delayed greenness during developmental leaf senescence (Rosado et al., 2019), indicating that phyB/PIF-mediated red-light signaling acts as a key regulatory module of dark-induced and developmental leaf senescence in many plant species.

While the role of phyB-mediated red light signaling in the regulation of dark-induced leaf senescence has been widely studied, this mechanism has also been shown to affect leaf senescence under light. Detached leaves of the *osphyB* knockout mutant turned yellow faster than those of WT plants when incubated in N-free liquid medium, and the accelerated leaf yellowing phenotype of *osphyB* leaves was recovered by the

supplementation with N sources such as potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3) (Piao et al., 2015), indicating that N availability is one of the key determinants for OsphyB-mediated regulation of leaf senescence under light conditions.

In Arabidopsis, phyB-mediated red-light signaling is involved in the promotion of phosphate (PO_4^{3-}) uptake *via* roots by enhancing the expression of high-affinity phosphate transporter genes, including *PHOSPHATE TRANSPORTER1;1* (*PHT1;1*) (Sakuraba et al., 2018). On the other hand, the expression of ammonium transporter genes, *AMT1;1*, *AMT1;2*, and *AMT2;1*, was upregulated in Arabidopsis seedlings grown under red-light illumination (Huang et al., 2015). Genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) analyses showed that HY5, a positive regulator of phyB-mediated red-light signaling (Gangappa and Botto, 2016), as well as PIF4, directly bind to the promoters of genes associated with the uptake and assimilation of N (Lee et al., 2007; Oh et al., 2012). Moreover, upon the exposure of plants to light, HY5 is translocated from shoots to roots, where it directly enhances the expression of *NRT2.1* (Chen et al., 2016). Considering the involvement of phyB-mediated red-light signaling in the acquisition of nutrients in Arabidopsis, it is highly likely that phyB-mediated red-light signaling also plays important roles in the regulation of N starvation-induced leaf senescence.

Involvement of NIGT1 transcription factors in the regulation of N starvation-induced leaf senescence

NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR1 (NIGT1) transcription factors act as negative regulators in nitrate inducible gene expression. In Arabidopsis, NIGT1 transcription factors directly repress *NRT2* genes and other N deficiency-inducible genes (Maeda et al., 2018; Kiba et al., 2018), and thus a gradual reduction in *NIGT1* transcript levels under N deficiency leads to the activation of N deficiency-inducible genes (Kiba et al., 2018).

Very recently, Tan et al. (2022) reported that *Malus domestica* HYPERSENSITIVE TO LOW PI-ELICITED PRIMARY ROOT SHORTENING1 (HRS1) HOMOLOG3 (*Md*HHO3), which is phylogenetically classified into NIGT1 family protein, directly represses *Malus domestica NRT2.1* (*MdNRT2.1*) transcript level, similar to Arabidopsis NIGT1s (Tan et al., 2022). Moreover, Arabidopsis and tobacco transgenic plants that overexpressing *MdHHO3* exhibited premature leaf yellowing phenotype, with the upregulation of senescence-associated genes, such as *NYC1*, *PAO*, and *SGR1*, under N deficiency (Tan et al., 2022), that NIGT1 transcription factors also act as a negative regulator in the N starvation-induced leaf senescence.

Involvement of microRNAs in the regulation of N starvation-induced leaf senescence

Small RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), are considered the key signaling molecules that regulate the expression of genes at the post-transcriptional level. In plants, miRNAs play important roles in the regulation of various environmental stress responses, including nutrient deficiency responses (Paul et al., 2015).

During N starvation, the accumulation of a number of miRNAs also changes in Arabidopsis and other plant species. Small RNA sequencing of Arabidopsis seedlings grown under N-sufficient and N-deficient conditions showed that the expression of more than 20 *miRNAs* significantly decreased, while that of several *miRNAs* increased (Liang et al., 2012). Among these miRNAs, *miR826* was strongly upregulated during N deficiency, and transgenic Arabidopsis plants overexpressing *miR826* or *miR5090*, which was identified from the complementary transcripts of *miR826*, exhibited better growth with delayed leaf yellowing under N-deficient conditions (He et al., 2014). On the other hand, the expression of *miR169* significantly decreased during N deficiency, and transgenic Arabidopsis plants overexpressing *miR169* exhibited accelerated leaf yellowing phenotype under N-deficient conditions (Zhao et al., 2011). *miR164s* are known to target the mRNA of *ORE1*, a central regulator of leaf senescence in Arabidopsis, to repress its expression at post-transcriptional level (Kim et al., 2009). During N deficiency, the expression levels of three *miR164s* (*miR164a*, *miR164b*, and *miR164c*) significantly decreased, while the *ORE1* transcript level was significantly elevated (Park et al., 2018), indicating that *miR164s* are involved in the suppression of N starvation-induced leaf senescence.

As described above, several N starvation-responsive miRNAs function in the regulation of N starvation-induced leaf senescence. Thus, it would be highly interesting to elucidate the roles of other N starvation-responsive miRNAs, as well as N starvation-responsive siRNAs, for further understanding of the regulatory mechanisms in N starvation-induced leaf senescence at the post-transcriptional level.

Conclusion and perspectives

N starvation-induced leaf senescence is a highly complex process finely controlled by several regulatory factors at different levels. To date, numerous genes associated with N starvation-induced leaf senescence have been identified, mostly in Arabidopsis. However, many more genes are expected to be involved in the regulation of N starvation-induced leaf senescence and to form a highly complex regulatory network. In recent years, several studies have attempted to identify the

genes associated with N deficiency responses using experimental approaches that employ big data, such as GWAS and gene co-expression analysis, providing new insights into the mechanisms of N deficiency responses. In the GWAS using the parameter of the reduction in chlorophyll content of 52 Arabidopsis accessions grown under N deficient conditions, several peaks potentially associated with N deficiency-induced leaf yellowing were identified, and several genes, including *NRT1.1*, *AGAMOUS-LIKE65* (*AGL65*), *ATP-BINDING CASSETTE G1* (*ABCG1*), and *INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 3* (*ITPK3*), were found near the peaks (Sakuraba et al., 2021b). As described in section 3.4, the significance of NRT1.1 in the regulation of N starvation-induced leaf senescence has been demonstrated. To dissect the gene regulatory network and identify novel genes associated with N deficiency responses in rice, Ueda et al. (2020) performed gene co-expression analysis and machine learning-based pathway inference using the transcriptome data of rice seedlings exposed to N-sufficient and N-deficient conditions (Ueda et al., 2020). Based on the results, several transcription factors were predicted to function as key regulators of the gene regulatory networks involved in N deficiency responses. In addition, transcription factors identified based on gene co-expression analysis and machine learning-based pathway inference also included OsNAC2, which acts as an enhancer of leaf senescence by controlling the accumulation of abscisic acid (ABA) (Mao et al., 2017), and OsWRKY23, which is used as a marker gene of leaf senescence (Han et al., 2020). Therefore, the transcription factors identified Ueda et al. (2020) most likely include key regulators of N starvation-induced leaf senescence. Functional characterization of each gene identified by the analyses using big data will further reveal the regulatory networks of N starvation-induced leaf senescence.

Recent studies revealed the significance of peptide hormones in the regulation of leaf senescence and nutrient starvation responses. In Arabidopsis, the small secreted peptide CLAVATA3/ESR-RELATED 14 (CLE14) functions in the suppression of leaf senescence by regulating the accumulation of reactive oxygen species (Zhang et al., 2022a). In Arabidopsis, CLE42 also acts as a negative regulator of leaf senescence by suppressing the biosynthesis of ethylene (Zhang et al., 2022b). Moreover, CLE42 showed functional redundancy with CLE41 and CLE44 in the suppression of leaf senescence: *cle41 cle42 cle44* triple mutant exhibited a strong early senescence phenotype (Zhang et al., 2022b). On the other hand, the root-to-shoot mobile peptide hormones C-TERMINALLY ENCODED PEPRIDEs (CEPs) and two CEP receptors (CEPRs) mediates N acquisition response accompanied by N-deficiency symptom to adapt to fluctuations in local N availability (Tabata et al., 2014). The involvement of these peptide hormones in the regulation of N starvation-induced leaf senescence is not yet investigated, therefore, examining this possibility is important for a better understanding of the molecular mechanisms underlying N starvation-induced leaf senescence.

While the molecular mechanisms underlying N starvation-induced leaf senescence have been studied mostly in the model plant Arabidopsis, the knowledge gained from Arabidopsis must be applied to crop plants. In the last two decades, the genome of a variety of crop plants has been sequenced (Goff et al., 2002; Schmutz et al., 2010; Sato et al., 2012), thus enabling the systematic analysis of plant biological processes, including N starvation-induced leaf senescence, by comparative genomics. Furthermore, owing to the recent advent of the CRISPR/Cas9 technology, which allows the modification of genomes without leaving behind any trace of foreign DNA (Jyoti et al., 2019), it is now possible to generate crop plants capable of displaying enhanced growth and high yield under a N-limited environment by modulating the function of gene(s) associated with N starvation-induced leaf senescence.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Zhonghai Li,
Beijing Forestry University, China

REVIEWED BY
Yongfeng Guo,
Tobacco Research Institute, Chinese Academy of Agricultural Sciences, China
Tomotsugu Koyama,
Suntory Foundation for Life Sciences, Japan

*CORRESPONDENCE
Hyobin Jeong
hyobinjeong@hanyang.ac.kr
Pyung Ok Lim
polim@dgist.ac.kr

†PRESENT ADDRESS
Hyobin Jeong,
Hanyang Institute of Bioscience and Biotechnology, Hanyang University, Seoul, South Korea;
Department of Life Science, College of Natural Sciences, Hanyang University, Seongdong-gu, Seoul, South Korea

‡These authors have contributed equally to this work

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Dynamic landscape of long noncoding RNAs during leaf aging in *Arabidopsis*

Jung Yeon Kim[1‡], Juhyeon Lee[1‡], Myeong Hoon Kang[1‡], Tran Thi My Trang[1], Jusung Lee[1], Heeho Lee[1], Hyobin Jeong[2,3*†] and Pyung Ok Lim[1*]

[1]Department of New Biology, Daegu Gyeongbuk Institute of Science and Technology (DGIST), Daegu, South Korea, [2]Genome Biology Unit, European Molecular Biology Laboratory (EMBL), Meyerhofstraße 1, Heidelberg, Germany, [3]Department of Life Science, College of Natural Sciences, Hanyang University, Seoul, South Korea

Leaf senescence, the last stage of leaf development, is essential for whole-plant fitness as it marks the relocation of nutrients from senescing leaves to reproductive or other developing organs. Temporally coordinated physiological and functional changes along leaf aging are fine-tuned by a highly regulated genetic program involving multi-layered regulatory mechanisms. Long noncoding RNAs (lncRNAs) are newly emerging as hidden players in many biological processes; however, their contribution to leaf senescence has been largely unknown. Here, we performed comprehensive analyses of RNA-seq data representing all developmental stages of leaves to determine the genome-wide lncRNA landscape along leaf aging. A total of 771 lncRNAs, including 232 unannotated lncRNAs, were identified. Time-course analysis revealed 446 among 771 developmental age-related lncRNAs (AR-lncRNAs). Intriguingly, the expression of AR-lncRNAs was regulated more dynamically in senescing leaves than in growing leaves, revealing the relevant contribution of these lncRNAs to leaf senescence. Further analyses enabled us to infer the function of lncRNAs, based on their interacting miRNA or mRNA partners. We considered functionally diverse lncRNAs including antisense lncRNAs (which regulate overlapping protein-coding genes), competitive endogenous RNAs (ceRNAs; which regulate paired mRNAs using miRNAs as anchors), and mRNA-interacting lncRNAs (which affect the stability of mRNAs). Furthermore, we experimentally validated the senescence regulatory function of three novel AR-lncRNAs including one antisense lncRNA and two mRNA-interacting lncRNAs through molecular and phenotypic analyses. Our study provides a valuable resource of AR-lncRNAs and potential regulatory networks that link the function of coding mRNA and AR-lncRNAs. Together, our results reveal AR-lncRNAs as important elements in the leaf senescence process.

KEYWORDS

leaf senescence, transcriptome (RNA-seq), Arabidopsis, long noncoding RNA, RNA-RNA interaction

Introduction

A leaf is a representative organ encompassing the fundamental characteristics of a plant. During the lifespan of a leaf, a series of physiological and functional shifts are observed, which end with senescence and death (Lim et al., 2007). At the early leaf developmental stages, the photosynthetic machinery is assembled *via* the biogenesis of chloroplasts and the synthesis of photosynthetic pigments, which in turn contribute to plant growth. After the maturation stage, leaves undergo organ-level senescence, which involves the orderly disassembly of subcellular organelles and macromolecules and the concomitant relocation of hydrolyzed molecules to actively growing organs such as developing seeds (for successful reproduction in annuals) or storage organs such as stems or roots (for the preparation of the next generation in perennials). Thus, despite its degenerative features, leaf senescence is critical for ensuring optimal offspring production and enhancing plant survival (Lim et al., 2007; Sasi et al., 2022).

Leaf senescence is triggered by an innate developmental program. However, this process is substantially affected by internal factors such as reproduction as well as external factors such as abiotic and biotic stresses. To ensure an integrated response to these internal/external factors, leaf senescence is tightly controlled by an intertwined network of developmental- or stress-associated pathways over time (Woo et al., 2019; Zhang et al., 2021). Attempts have been made to dissect the molecular mechanisms underlying leaf senescence through the identification of senescence-altered mutants and the functional characterization of senescence regulatory genes in *Arabidopsis* as well as in agriculturally important crops, revealing dozens of key regulatory molecules including transcription factors, epigenetic regulators, regulatory microRNAs (miRNAs), protein-modifying molecules, and small secretory peptides, thus expanding our knowledge of how leaf senescence is fine-tuned at the chromatin, transcriptional, post-transcriptional, translational, and post-translational levels (Woo et al., 2019; Zhang et al., 2022). More recently, the temporal dynamics of age-related regulatory networks and molecular mechanisms linking environmental signals with innate senescence pathways have been further explored using multi-omics technologies, together with computational biology tools and extensive biochemical and molecular genetic analyses (Woo et al., 2016; Lyu et al., 2017; Kim et al., 2018).

Previous transcriptome analyses, which mainly focused on protein-coding genes, revealed a detailed chronology of leaf senescence-associated physiological processes, highlighting the transcriptome as a significant molecular signature of leaf senescence (Breeze et al., 2011; Woo et al., 2016) Although these studies are worthy to infer key regulatory elements and pathways, the analyses remain limited to unraveling the hidden layers of leaf senescence-related gene regulatory networks.

Long noncoding RNAs (lncRNAs) are long transcripts (>150 nt) with poor protein-coding potential (<50 amino acids) and are emerging as important modulators of gene expression in diverse biological processes in animals and plants (Dinger et al., 2008; Chen et al., 2020; Statello et al., 2021). The genomic origins and biogenesis processes vary widely among lncRNAs, ranging from intergenic regions, introns of annotated genes, or to the antisense strand of neighboring protein-coding genes (referred to as natural antisense transcripts [NATs]). The lncRNAs are a functionally heterogeneous group of RNA molecules that regulate gene expression by interacting with specific DNAs, RNAs, or proteins through *in cis* or *in trans* mechanisms involving chromatin remodeling (Heo and Sung, 2011), RNA processing (Bardou et al., 2014), RNA stabilization (Ma et al., 2021a), and translational regulation (Jabnoune et al., 2013). The lncRNAs also act as decoys of miRNA or RNA-binding proteins (Seo et al., 2019; Liu et al., 2022), generate miRNAs (Augoff et al., 2012), or are translated into small open reading frames (sORFs) (Romero-Barrios et al., 2018).

Genome-wide transcriptome analyses in plants revealed thousands of lncRNAs, which are differentially expressed in response to abiotic and biotic stresses. However, only limited numbers of plant lncRNAs have been functionally analyzed. For example, *COLD INDUCED LONG ANTISENSE INTRAGENIC RNA* (*COOLAIR*) and *COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*) are involved in vernalization-mediated regulation of flowering (Heo and Sung, 2011); *ELF18-INDUCED LONG-NONCODING RNA1* (*ELENA1*) regulates the expression of *PATHOGENESIS-RELATED1* (*PR1*) gene, which encodes a key plant immunity related protein (Seo et al., 2019).; *INDUCED BY PHOSPHATE STARVATION1* (*IPS1*) regulates phosphate homeostasis as an endogenous target mimic (eTM) of *miR399* (Franco-zorrilla et al., 2007); *HIDDEN TREASURE 1* (*HID1*) affects photomorphogenesis by regulating the expression of the *PHYTOCHROME-INTERACTING FACTOR 3* (*PIF3*) gene (Wang et al., 2014); *AUXIN REGULATED PROMOTER LOOP* (*APOLO*) regulates *PINOID* (*PID*) expression by modulating chromosome loop dynamics, thereby affecting auxin signaling (Ariel et al., 2014). These results indicate that lncRNAs serve as crucial regulators of plant development and stress responses. Recently, the importance of lncRNAs in leaf senescence has been also explored in tomato and rice (Huang et al., 2021; Li et al., 2022). However, their roles in the regulation of leaf senescence still remain poorly understood. One of the major obstacles that inhibit mechanistic studies of leaf senescence-related lncRNAs is the lack of a genome-wide systematic analysis of lncRNAs.

In this study, we performed comprehensive analyses of RNA-seq data collected from *Arabidopsis thaliana* leaves at different developmental stages, leading to the identification of age-related lncRNAs (AR-lncRNAs). Multiple types of analyses were conducted including the characterization of AR-lncRNAs and prediction of their potential target genes by linking the functions of protein-coding RNAs to those of AR-lncRNAs. Knockout mutations of three AR-lncRNAs resulted in altered leaf senescence phenotypes, validating the regulatory role of these AR-lncRNAs in senescence process. Our results will serve as a useful resource and framework for further functional and

mechanistic studies to reveal the detailed regulatory role of lncRNAs in *Arabidopsis* leaf senescence.

Materials and methods

Data source

The reference genome sequence and transcriptome annotation GTF files of *Arabidopsis thaliana* were downloaded from The Arabidopsis Information Resource (TAIR10.47). The previously published strand-specific total RNA-seq and small RNA (sRNA)-seq data derived from the leaves of *Arabidopsis thaliana* ecotype Columbia (Col-0) (Woo et al., 2016) were used in this study.

Identification of novel lncRNAs in *Arabidopsis*

The TruSeq adapter sequences were removed using Trimmomatic to obtain clean reads (average Phred quality score ≥ 20), which were then aligned to the *Arabidopsis* reference genome (TAIR10.47) using TopHat2, with a default parameter (mismatch ≤ 2 nt) (Kim et al., 2006a; Bolger et al., 2014). To predict novel transcripts, mapped reads from each bam file were assembled using Cufflinks with the -M parameter (Trapnell et al., 2010). The resultant GTF file was merged with the annotated lncRNAs from TAIR10.47 using cuffmerge. Read counts were then generated using HTseq-count, and expression levels were estimated as transcripts per million (TPM) (Anders et al., 2015). To select *Arabidopsis* lncRNAs, only transcripts with Cufflinks class codes 'u' (intergenic transcripts), 'x' (exonic overlap with reference sequence on the opposite strand), and 'i' (transcripts entirely within the intron) were retained. Then, short transcripts (<150 nt) and low-abundance transcripts (maximum TPM [TPMmax] < 1) were removed. Unannotated transcripts were named using the Cufflinks annotation (XLOC_).

lncRNA characterization and sORF detection

Small RNA and their precursors were predicted based on the previously published small RNA-seq data of aging leaves (Axtell, 2013b; Woo et al., 2016) using Shortstack with default parameters. Then, the ribosome footprint sequencing (Ribo-seq) data of the leaves of 3-week-old plants (Lukoszek et al., 2016) were used to predict the ribosome-associated lncRNAs (ribo-lncRNAs). Subsequently, putative translated sORFs were predicted by calculating the ribosome release score (RRS), which indicates whether the ribosome footprint decreases after the termination codon of sORFs (≥30 nt, 10 amino acids) (Guttman et al., 2014; Bazin et al., 2017).

Identification and analysis of differentially expressed genes

Differentially expressed transcripts were identified using the DESeq2 method (Love et al., 2014). Enrichment analysis of Gene Ontology (GO) terms was performed using DAVID v6.8 (Huang et al., 2008).

Prediction of RNA–RNA interactions

Interaction energies between AR-lncRNAs and mRNAs were calculated from the FASTA file of TAIR10.47 using RIBLAST 1.1.1 (Fukunaga and Hamada, 2017a). with the following thresholds: interaction energy < -16 kcal/mol, and interaction length ≥ 15 nt. Pearson correlation coefficients of the predicted AR-lncRNA and mRNA pairs detected throughout the leaf lifespan were calculated using the average TPM values.

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) is the wildtype strain for all mutants. Plants were grown in an environmentally controlled growth room (Korea Instruments, Korea) at 22℃ under 16h light:8h dark photoperiod and photosynthetic photon flux density of 130μmol $m^{-2}s^{-1}$. The Arabidopsis transfer DNA (T-DNA) insertion lines, SALK_100875 (*at5g01595*), SALK_151843 (*atfer1*) SALK_124431C (*at1g33415*), and SALK_135316 (*at2g14878*) were obtained from Arabidopsis Biological Resource Center. The genotype of each mutant line was confirmed by PCR-based genotyping.

Leaf senescence assay

For developmental leaf senescence, the third and fourth leaves of each plant, at the indicated age, were harvested at 4 to 5h after light-on. For dark-induced senescence, the 14-d-old third and fourth leaves of each plant were detached and floated upside down on 3mM MES buffer (pH 5.7) in 24-well plates, which were completely wrapped with aluminum foil. The photochemical efficiency (F_v/F_m) ratio of photosystem II was measured by a Walz IMAGING-PAM machine.

RNA isolation and quantitative RT-PCR analysis

Total RNA was extracted from third and fourth rosette leaves were extracted with TRIzol and treated with DNase I (Ambion). cDNA was synthesized using the ImPromII™ system (Promega) reverse transcription kit following the manufacturer's

instruction. The extracted cDNA was used for semi-quantitative and quantitative real time Polymerase chain reaction (RT-PCR and qRT-PCR). qRT-PCR analysis was carried out to determine the gene expression levels (CFX96 system, Bio-Rad). Transcript abundances of target genes were analyzed by the comparative threshold method, with *ACTIN2* (*AT3G18780*) as the internal control. For visualization of amplified cDNA band in RT-PCR, 10μl of the amplified product was run in 0.8% agarose gel containing ethidium bromide.

Results

Genome-wide identification of lncRNAs in *Arabidopsis* leaves

Previously, we generated multidimensional transcriptome data of 4-d- to 30-d-old *Arabidopsis* leaves, and characterized the regulatory features of leaf senescence (Woo et al., 2016) (Supplementary Data 1). In this study, we re-analyzed our previously generated RNA-seq dataset to assess the role of lncRNAs in the development of leaf organs. A total of 700 million reads were aligned against TAIR10 using TopHat2 (Kim et al., 2006a), and the mapped reads were assembled using Cufflinks (Trapnell et al., 2010). This led to the identification of 59,368 unique transcripts, corresponding to 31,405 gene loci. Among the 59,368 unique transcripts, 50,465 were previously annotated as protein-coding transcripts or as noncoding RNAs such as housekeeping RNAs (e.g., tRNAs, rRNAs, small nuclear RNAs, and small nucleolar RNAs) and miRNA precursors. In addition to these 50,565 transcripts, 4,815 short transcripts (length < 150 nt) and 3,262 low-abundance transcripts (TPMmax < 1) were excluded from the dataset of unique transcripts. Subsequently, 54 transcripts showing protein-coding potential, as determined by the Coding Potential Calculator (CPC) (Kang et al., 2017), were further eliminated. Thus, 771 lncRNAs were identified, of which 539 were previously annotated and 232 were novel (Figure 1A, Supplementary Data 2).

Based on their predicted functions, the 771 lncRNAs were classified into the following three categories: 1) ribo-lncRNAs, lncRNAs that potentially encode an sORF or are associated with the stability and translation of their cognate mRNAs *in trans*; 2) sRNA precursors, lncRNAs that generate precursors of small RNAs such as miRNAs and *trans*-acting or phased small interfering RNAs (tasiRNAs/phasiRNAs); and 3) other canonical lncRNAs not included in the first two categories (Figure 1A). Ribo-lncRNAs (145/771 [18.78%]) were predicted using ribo-seq data generated from the leaves of 3-week-old *Arabidopsis* plants (Lukoszek et al., 2016). Translated sORFs are often hidden among ribo-lncRNAs (93/145 [64.14%]). Potential sORFs encoding > 10 amino acids from ribo-lncRNAs were recognized using two analytics: RRS, which evaluates the decrease in ribosome footprint number after termination codons (Bazin et al., 2017); and getorf, which finds and outputs the sequence of ORFs in nucleotide sequences (Rice et al., 2000). This analysis allows to identify putative lncRNA-encoded sORFs with RRS ≥ 0.9 (29). The remaining ribo-lncRNAs might be involved in the stabilization and translation of their cognate mRNAs or in the *trans*-regulation of mRNAs. LncRNAs capable of generating the precursors of 21–22-nt long sRNAs (34/771 [4.40%]) were predicted based on the small RNA-seq data (Axtell, 2013a; Woo et al., 2016) (Figure 1A). Furthermore, we confirmed the RNA-seq read coverage of representative lncRNAs in each category (ribo-lncRNAs, sRNA precursors, and canonical lncRNAs) using gene viewer (Figure 1B).

In addition to the classification of 771 lncRNAs based on their predicted functions, we further classified these lncRNAs according to their genomic locations. Six genomic location-based categories of lncRNAs were identified: intergenic (325 out of 771 lncRNAs, 42.15%), antisense (305 [39.55%]), divergent (74 [9.60%]), convergent (96 [12.45%]), divergent & convergent (31[4.02%]), and intronic (3 [0.39%]) (Figure 1C). We then characterized the features of lncRNAs, such as average length, exon number, isoform number, and expression level, in each category, and compared the results with the features of protein-coding transcripts. The lncRNAs were shorter (average length = 878.757 bp) and contained fewer exons (average exon number = 1.72) than coding transcripts (2215.58 bp and 4.6 exons, respectively) ($p < 0.0001$, Mann-Whitney *U*-test, two-tailed) (Figures 1D, E). On the other hand, the number of isoforms of lncRNAs (1.34) was comparable with that of coding RNAs (1.6) (Figure 1F). The median expression levels of lncRNAs (average TPM along leaf age) were significantly (11-fold) lower than those of coding transcripts ($p < 0.0001$, Mann-Whitney U-test, two-tailed) (Figure 1G). These results are consistent with those of previous studies, which identified lncRNAs involved in other biological processes (Di et al., 2014; Tsai et al., 2022). The RNA-seq read coverage of the novel lncRNAs identified in this study was confirmed using gene viewer (Supplementary Figure 1A), and their expression levels were validated through the RT-PCR analysis of eight randomly-selected transcripts (Supplementary Figures 1B, C).

Identification of AR-lncRNAs

The functional transition of leaves during aging inspired us to examine the dynamic landscapes of lncRNAs. Of the 771 lncRNAs identified in this study, 446 (57.8%) were differentially expressed during leaf aging, as examined by DEseq2 (Love et al., 2014) (|log2(fold change)| ≥ 1, adjusted *p*-value [p_{adj}] ≤ 0.05) (Figure 2A). Among the AR-lncRNAs, 192 and 292 lncRNAs showed dynamic changes of their expressions during the early biogenesis period (from growth [G] to maturation [M], 4–18 d)

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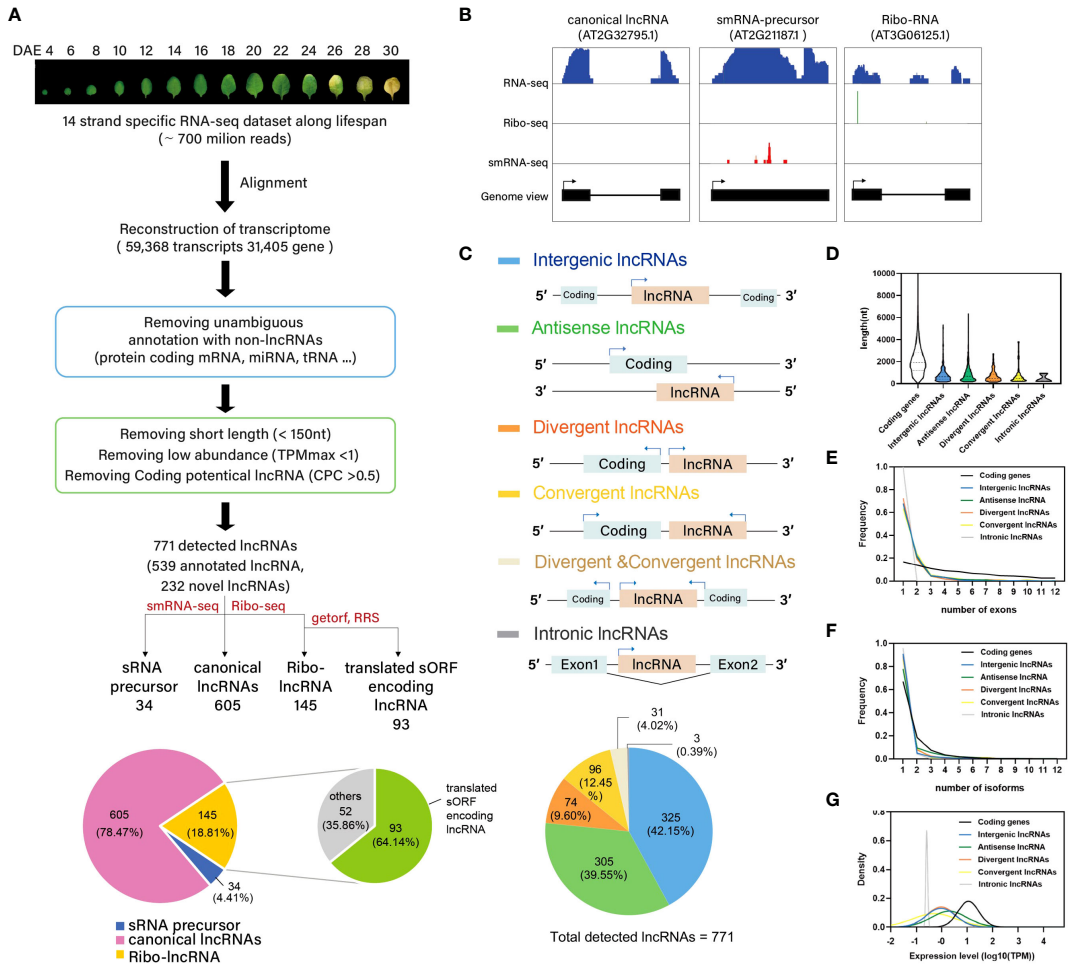

FIGURE 1
Genome-wide identification of lncRNA in *Arabidopsis*. **(A)** Pipeline for the systematic identification of lncRNAs in *Arabidopsis*. Note that there are 13 detected lncRNAs which were belonging to both sRNA precursor and Ribo-lncRNA. **(B)** Normalized read coverage of representative lncRNAs in each category, as measured by RNA-seq, sRNA-seq, and ribo-seq. **(C)** Classification of lncRNAs based on their genomic location. **(D)** Violin plot showing the length distribution of lncRNAs. **(E)** Frequency line plot showing the distribution of exon numbers in different lncRNAs. **(F)** Frequency line plot showing the distribution of isoform numbers of different lncRNAs. **(G)** Distribution curve of average expression levels of lncRNAs.

and late degeneration period (from M to senescence [S], 16–30 d), respectively, indicating that lncRNAs play important roles in leaf development. The majority of AR-lncRNAs were intergenic (44.39%), followed by antisense (37.44%), divergent (9.86%), convergent (12.11%), divergent & convergent (4.04%), and intronic (0.22%). The proportions of genomic location of AR-lncRNAs were similar to that of detected lncRNAs, and not significantly different between the G→M and M→S transitions (Figure 2B). Notably, the number of senescence-associated AR-lncRNAs (M→S) was greater than that of biogenesis associated-lncRNAs (G→M), suggesting that lncRNAs are more relevant to the leaf senescence process than to the leaf biogenesis process.

Differentially expressed lncRNAs were categorized into six major clusters, including three upregulated (U1–U3) and three downregulated (D1–D3) clusters, based on their expression kinetics over time, which represents 97% of the AR-lncRNAs (Figures 2C, D). We also examined the temporal expression profiles of lncRNAs showing age-dependent changes in transcript levels during the G→M and M→S transitions. During the G→M transition, the numbers of upregulated and downregulated AR-lncRNAs were similar (54% and 46%, respectively) (Figure 2E). Intriguingly, the majority of AR-lncRNAs (69%) were upregulated during the M→S transition. AR-lncRNA were preferentially upregulated, regardless of their genomic location (Figure 2E). Canonical lncRNAs were also preferentially upregulated, but rather similar numbers of ribo-lncRNAs and sRNA-precursor lncRNAs were up- and downregulated. The expression patterns of four randomly-selected AR-lncRNAs were verified by RT-PCR (Figure 2F).

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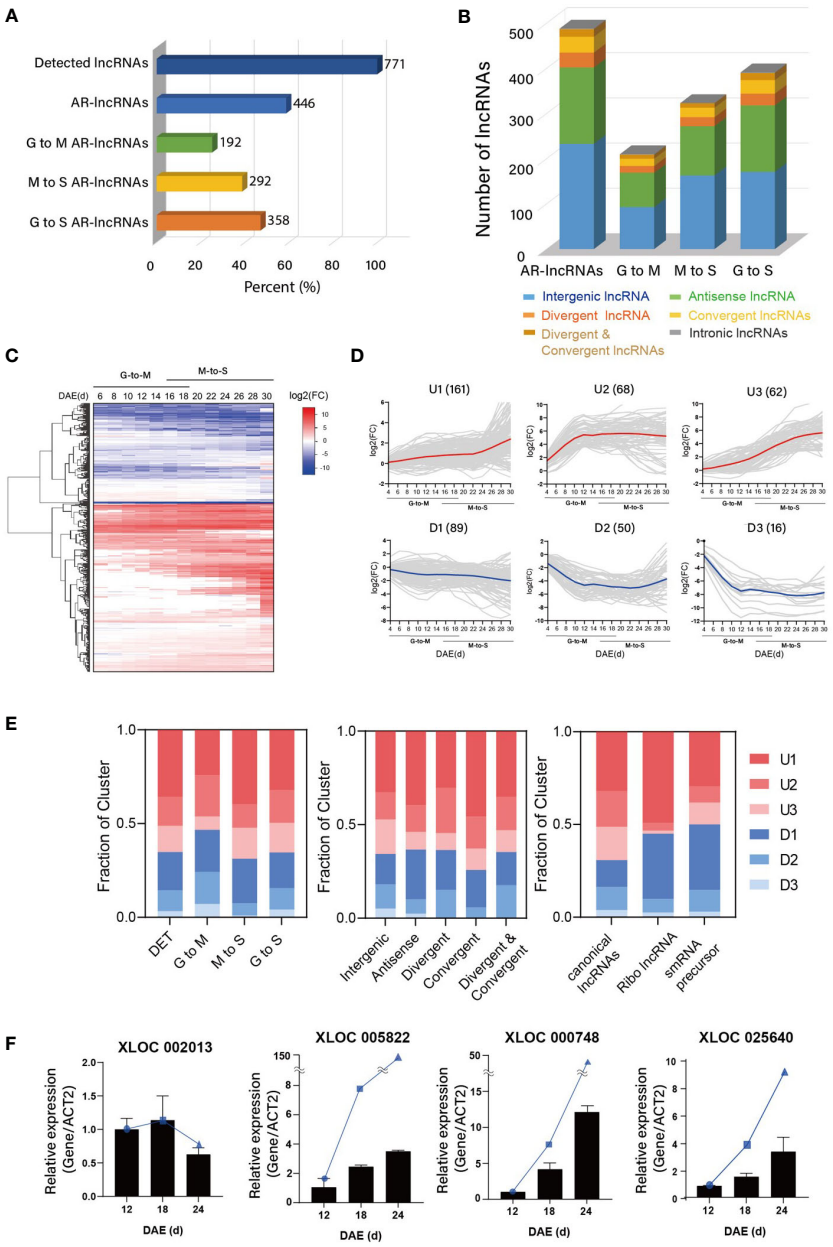

FIGURE 2
Identification of age-related lncRNAs (AR-lncRNAs) in *Arabidopsis*. **(A)** Proportions of lncRNAs detected in the reconstructed leaf transcriptome and differentially expressed in aging leaves (fold change ≥ 2, p <= 0.05). The leaf lifespan was divided into two developmental stages, G-to-M (Growth to Maturation, 4–18 d) and M-to-S (Maturation to Senescence, 16–30 d). Some of G-to S AR-lncRNAs are overlapped with G-to-M AR-lncRNAs or M-to-S AR-lncRNAs. **(B)** Proportions of AR-lncRNAs at different genomic locations with respect to the nearest protein-coding gene. **(C)** Heat maps showing the expression of AR-lncRNAs over the entire leaf lifespan. Rows are ordered based on hierarchical clustering. Color bar represents the gradient of log2(fold change) values relative to the 4-DAE time point. DAE: days after emergence. **(D)** Changes in AR-lncRNA transcript levels in aging leaves, as shown by k-means clustering. Six major clusters (upregulated, U1–U3; downregulated, D1–D3) were detected, depending on AR-lncRNA expression patterns. **(E)** Proportions of major AR-lncRNAs in different categories established based on the different developmental stages of leaves and the genomic locations and functions of AR-lncRNAs. **(F)** Expression analysis of novel AR-lncRNAs at three timepoints by qRT-PCR. Error bars represent the standard error of mean (SEM; n = 3). The blue circles, squares, and triangles means the TPM value of each lncRNA at indicated leaf ages (12d, 18d, 24d) from RNA-seq.

Studies show that lncRNAs localize to various subcellular organelles, and regulate gene expression at various levels (transcriptional, post-transcriptional, and translational) (Bridges et al., 2021). Therefore, knowledge of the subcellular localization patterns of lncRNAs would provide information for inferring their gene regulation mode. We determined the subcellular localization of each AR-lncRNA by analyzing the publicly available transcriptome data of the cytosolic and nuclear fractions of 2-week-old *Arabidopsis* seedlings (Zhao et al., 2018). Of the 287 AR-lncRNAs identified in these transcriptomes, 211 (73.52%) were predominantly present in the nuclear fraction, whereas only 76 (26.48%) were enriched in the cytosolic fraction (Supplementary Figures 2A, B and Supplementary Data 5). This pattern was robust, regardless of the functional categories of AR-lncRNAs. For instance, both sRNA-precursor lncRNAs (19/23 [81.61%]) and sORF-encoding lncRNAs (56/93 [60.22%]) were more abundantly localized in the nuclear fraction. To confirm this result, we performed qRT-PCR on six randomly-selected lncRNAs using cDNA isolated from the nuclear and cytosolic fractions of 2-week-old seedlings, which validated the subcellular localization of all, but one, lncRNAs (Supplementary Figure 2C).

Leaf development is an integrated response of plants to an innate developmental program and environmental stress responses. Thus, some of the genes involved in leaf senescence are expected to control environmental responses. We therefore investigated whether AR-lncRNAs are regulated by stress responses using the previously published transcriptome data of ABA-, drought-, and cold-treated *Arabidopsis* (Zhao et al., 2018). Among the lncRNAs differentially expressed by the ABA, drought, or cold treatment, a large proportion (68/102 [66.7%], 72/112 [64.3%], and 70/127 [55%], respectively) was also affected by the developmental age, suggesting an extensive overlap between leaf senescence and stress responses. This result is consistent with that of the previous study on protein coding mRNAs (Zhao et al., 2018) (Supplementary Figures 3A, B and Supplementary Data 4).

Identification of antisense lncRNAs overlapped with neighboring protein-coding genes and functional validation of putative lncRNA in the regulation of leaf senescence

Expression levels of some lncRNAs are significantly correlated with those of their neighboring protein-coding genes. Several lncRNAs are also known to control the expression of nearby genes (Statello et al., 2021), suggesting that these lncRNAs act as *cis*-regulators of genes. Thus, the positional relationship between lncRNAs and mRNAs in the genome would be important for predicting the lncRNA-controlled regulation of nearby genes. To infer the effect of lncRNAs on the expression of neighboring genes in aging leaves, we estimated the degree of co-expression between AR-lncRNAs and their adjacent protein-coding genes by calculating the Pearson correlation coefficients (PCCs). The co-expression of all pairs of age-related protein-coding genes was also analyzed for comparison. Notably, the PCC between pairs of antisense AR-lncRNAs and overlapping protein-coding genes was significantly higher than that between overlapping protein-coding gene pairs ($p < 0.05$, Mann-Whitney U-test, two-tailed) (Figure 3A). This result implies that antisense AR-lncRNAs act as *cis*-regulators of adjacent genes during leaf aging. Of the 168 antisense AR-lncRNAs, the TPM fractional density of 72 antisense AR-lncRNAs and their overlapping protein-coding genes pairs (PCC > 0.7) was visualized as a heatmap (Figure 3B). Gene ontology biological process (GOBP) enrichment analysis revealed that the neighboring protein-coding genes of antisense lncRNAs were significantly enriched by cytokinin catabolic/metabolic process, flavonoid glucuronidation, defense response, and oxidation-reduction processes (Figure 3C). Cytokinin is a representative hormone that negatively regulates leaf senescence in plants. The expression of cytokinin biosynthetic genes decreases, while that of cytokinin degradation genes increases during leaf senescence in *Arabidopsis* (Buchanan-wollaston et al., 2003; Breeze et al., 2011; Statello et al., 2021). Genes encoding two *SOB five-like* (*SOFL*) genes, *AtSOFL1* and *AtSOFL2*, which act as positive regulator of cytokinin levels and cytokinin-mediated development including longevity (Zhang et al., 2009), are found to be overlapped with *AT1G26210* and *AT1G26208* AR-lncRNAs, respectively. Antisense AR-lncRNA *AT3G63445* is overlapped with *CYTOKININ OXIDASE* (*CKX*) *6* that catalyzes the degradation of cytokinin.

Defense responses are also one of the typical age-associated biological processes (Kus et al., 2002; Mao et al., 2017). *FERRITIN1* (*FER1*), overlapped with antisense AR-lncRNA *AT5G01595*, plays a role in iron hemostasis and accumulates upon exposure to oxidative stress or to pathogen attack, as well as developmental factor. Mutation of *FER1* causes earlier onset of leaf senescence (Murgia et al., 2007). So, it is likely that *AT5G01595* lncRNA might play a role in leaf senescence possibly through modulating *FER1*. To validate the functional role of *AT5G01595*, the senescence phenotype of the third and fourth leaves of knockout line (*at5g01595*) and wild-type (Col-0) plants during age-dependent natural senescence was compared (Figure 3D). Initiation of leaf yellowing, which is an indicator of chloroplast senescence in mesophyll cells, occurred earlier in *at5g01595* than in Col-0. The photochemical efficiency (F_v/F_m), a representative physiological marker of leaf senescence, also declined rapidly. The early leaf senescence phenotype of *at5g01595* was further confirmed by analyzing the expression of the *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*) and *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB2*), the molecular markers of leaf senescence (Supplementary Figures 4B, C). We then tested the effect of *AT5G01595* antisense lncRNA on the expression of *FER1*. The result showed that *FER1* transcript level was significantly lower in *at5g01595* than in Col-0 leaves (Figure 3D). These results

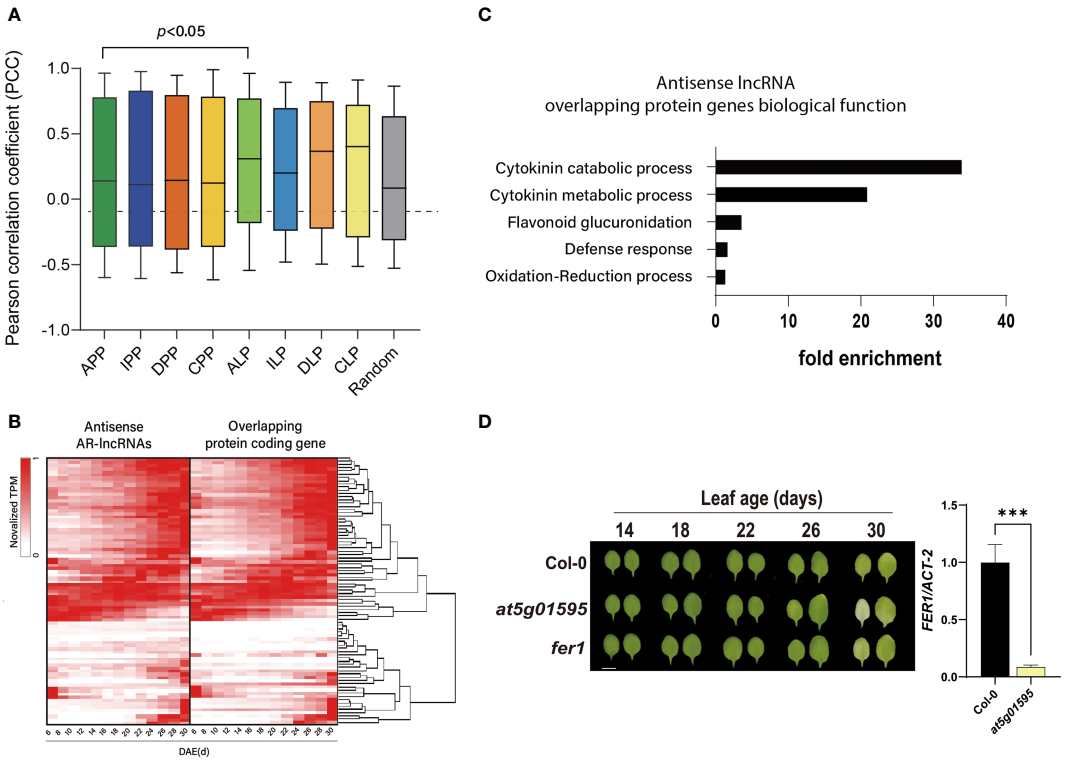

FIGURE 3
Expression correlation of antisense AR-lncRNAs and overlapping protein-coding genes during the leaf lifespan. **(A)** Box-plot displaying the expression correlation between AR-lncRNAs and adjacent genes. PCC: Pearson correlation coefficient; APP: overlapping protein-coding gene pairs; IPP: protein-coding genes and non-overlapping protein-coding gene pairs; DPP: divergent protein-coding gene pairs; CPP: convergent protein-coding gene pairs; ALP: antisense AR-lncRNAs and overlapping protein-coding genes; ILP: intergenic AR-lncRNAs and non-overlapping adjacent genes; DLP: divergent AR-lncRNAs and adjacent genes with a common promoter region; CLP: convergent AR-lncRNAs and adjacent genes within 1,000 bp; Random: random pairs. Central lines represent the mean. Whiskers represent the maximum and minimum values. Statistical difference is indicated by p-value (Mann- Whitney U-test. **(B)** Heat maps representing the expression pattern of antisense AR-lncRNAs and overlapping protein-coding genes. Rows are ordered based on hierarchical clustering. Columns indicate the number of days after emergence (DAE). Color bar shows the fraction density during the leaf lifespan. **(C)** Gene ontology biological process [GOBP of protein-coding genes overlapped with antisense AR-lncRNAs ($p < 0.05$)]. Fold enrichment represents the ratio of the proportion of input genes involved in GOBP and the proportion of genes in the given GOBP in involved in the background. **(D)** Phenotype of Col-0, *at5g01595* and *fer1* during developmental leaf senescence (left) and expression level of *FER1* mRNA was measured by qRT-PCR (right). Data in the right panel represent the mean of 3 biological replicates, and error bars represent SD (n = 3). Asterisks indicate significant differences (t -test; ***, $p < 0.001$).

indicate that *AT5G01595* potentially contributes to leaf senescence by modulating *FER1*.

Together, these findings suggest that leaves might utilize antisense lncRNAs as a regulatory program for controlling biological processes, particularly cytokinin-related processes and defense responses, throughout its lifespan.

Identification of the potential regulatory network involving competitive endogenous AR-lncRNAs.

The lncRNAs can regulate mRNAs by sequestering specific miRNAs and mimicking their target recognition sequence in organisms. We searched for potential competitive endogenous RNAs (ceRNAs) involved in lncRNA–miRNA–mRNA interactions. We first integrated the TarDB (Liu et al., 2021) and TarBase (Karagkouni et al., 2018) datasets to search for mRNA–miRNA interactions, and StarBase (Li et al., 2014) to search for lncRNA–miRNA interactions in *Arabidopsis*. Using the hypergeometric test, potential ceRNA sets (lncRNA–miRNA–mRNA) were identified (Figure 4A) by evaluating the significance of interacting miRNAs shared by both mRNAs and lncRNAs ($p < 0.05$); these shared miRNAs were used as a junction. Among the potential ceRNA sets, we further narrowed down high-confidence ceRNA sets by calculating the PCCs of lncRNAs and their cognate mRNAs. The selection of lncRNAs and mRNAs with PCC > 0.7 led to the identification of 602 positively-correlated ceRNA sets (Figure 4A and Supplementary Data 6). Eleven of the identified lncRNAs that paired with ceRNAs would likely compete with

miRNAs involved in leaf development or leaf senescence, such as miR156, miR164, and miR169 (Figure 4B). Among these miRNAs, miRNA164, a negative regulator of leaf senescence, is known to mediate the cleavage of *ORESARA1* (*ORE1*), which induces cell death and leaf senescence (Kim et al., 2009). One of the AR-lncRNAs, *AT4G36648*, was identified as a target of *miRNA164* in this analysis, which involves in the ceRNA set linking *AT4G36648-miRNA164-ORE1*. Both *AT4G36648* and *ORE1* showed a rapid change in expression at the late degeneration stage. A strong positive correlation between *AT4G36648* and *ORE1* (PCC = 0.99) supports the presence of ceRNA that anchors these transcripts through *miRNA164*. Moreover, *AT4G36648* was expressed during the M→S transition, which suggests the regulatory role of this ceRNA in leaf senescence. We also identified *AT5G23410-miR169-NUCLEAR FACTOR Y* (*NF-Y*) as ceRNA set. The expression level of the AR-lncRNA *AT5G23410*, followed by that of *miR169*-targeted *NFYA5*, which is known to modulate ABA-dependent stress responses (Li et al., 2008), was induced during aging. In this ceRNA set, the expression level of *AT5G23410* was highly correlated with that of *NFYA5* (PCC = 0.90). ABA is one of the hormones accelerating leaf senescence. Thus, this module is likely to be involved in mediating crosstalk between leaf senescence and stress responses. The AR-lncRNA *AT1G26208* was identified as an interacting partner of *miRNA156*, which inhibits the action of *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*). Both *miRNA156* and *SPL* form a regulatory module to control age-dependent developmental transition as well as abiotic and biotic stress responses (Wang et al., 2009; Ma et al., 2021b). Similar to the above two ceRNA sets, the age-dependent expression levels of AR-lncRNA *AT1G26208* and *SPL10* were highly correlated (PCC = 0.98), implying that the AR-lncRNA *AT1G26208* modulates age-dependent pathways or integrates aging cues with stress responses.

Next, we reconstructed the ceRNA (lncRNA–miRNA–mRNA) interaction network. This network is composed of 106 mRNAs differentially expressed during aging, and 27 AR-lncRNAs which are commonly targeted by 38 miRNAs (Figures 4C, D and Supplementary Data 6). To predict the potential function of AR-lncRNAs comprising the ceRNA network, the interacting mRNAs in the ceRNA network were subjected to the GOBP enrichment analysis. These genes were over-represented by the regulation of transcription and leaf senescence, suggesting that AR-lncRNAs perform an important regulatory role during leaf aging by participating in the ceRNA network (Figure 4E).

Identification of potential AR-lncRNAs interacting with mRNAs and functional validation of two lncRNAs in the regulation of leaf senescence

Numerous lncRNAs regulate gene expression by directly interacting with target mRNAs and affecting their stability or processing (Bardou et al., 2014; Sebastian-delaCruz et al., 2021). To identify putative mRNA-lncRNA pairs, we utilized RIBLAST, a computational tool used to predict comprehensive lncRNA–RNA interactions based on the seed-and-extension approach, where a target prediction was experimentally validated (Kretz et al., 2013; Fukunaga and Hamada, 2017b). We first calculated the interaction energy between the AR-lncRNA and mRNA sequences in the TAIR10 database, and then selected RNA segment pairs with < -16 kcal/mol interaction energy in 15 ≥ nt. This analysis led to the identification of 316,475 AR-lncRNA-mRNA pairs. These interacting pairs were further narrowed down based on the PCC values, resulting in the identification of highly co-expressed AR-lncRNA and target mRNA pairs (|PCC| ≥ 0.9) during the leaf lifespan. Through this analysis, we obtained 2,220 putative interactions among 446 AR-lncRNAs (Figure 5A and Supplementary Data 7). To explore AR-lncRNAs associated with leaf senescence, we searched for pairs of AR-lncRNAs and mRNAs whose genes are known to be involved in the regulation of leaf senescence (Supplementary Data 8) (Li et al., 2020). The predicted regulatory modules were further validated through the characterization of loss-of-function mutants. In this study, we focused on two AR-lncRNAs: *AT1G33415* and *AT2G14878* (Figures 5B, C).

Autophagy is required for nutrient recycling during leaf senescence. In *Arabidopsis*, Autophagy9 (APG9) is known to regulate the formation of autophagosomes, which are crucial for autophagy process, from the endoplasmic reticulum (Zhuang et al., 2017). The expression of *APG9* is markedly upregulated during leaf senescence, and the *apg9* mutant exhibits precocious senescence phenotypes, indicating that APG9 plays a negative role in leaf senescence (Zhuang et al., 2017). Given that *AT1G33415* interacts with *APG9* and the corresponding genes are highly co-expressed (Figure 5B), we decided to evaluate the involvement of *AT1G33415* in the regulation of leaf senescence.

Firstly, we compared the yellowing phenotype of the third and fourth leaves of mutant (*at1g33415*) and wild-type (Col-0) plants during age-dependent natural senescence. The progression of leaf yellowing occurred more rapidly in the mutant than in the wild type (Figure 5D). The early leaf senescence phenotype of *at1g33415* was confirmed by measuring the photochemical efficiency (F_v/F_m), which declined rapidly in *at1g33415* (Figure 5E). We also conducted a dark-induced leaf senescence assay using detached leaves. Similar to the leaf phenotype observed during age-dependent senescence, dark-induced senescence symptoms (leaf yellowing and F_v/F_m decline) were also accelerated in the leaves of *at1g33415* (Figures 5G, H). *SAG12* expression was also analyzed for confirming the senescence phenotype (Supplementary Figures 5A, B). We then examined the expression level of *APG9* in mature Col-0 and *at1g33415* leaves. The results showed that *APG9* transcript levels were significantly lower in *at1g33415* than in Col-0 leaves (Figure 5F). These results imply that *AT1G33415* potentially contributes to

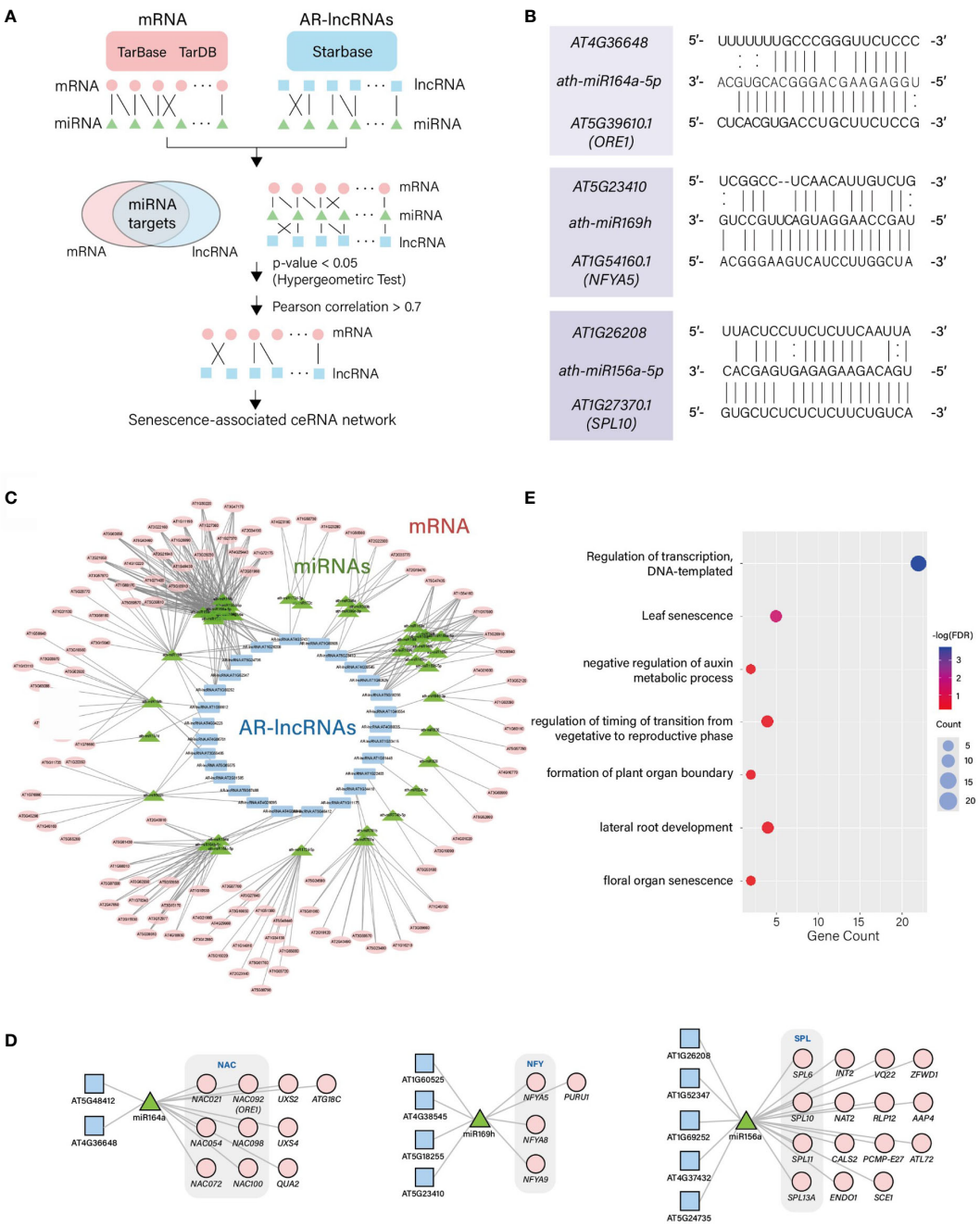

FIGURE 4
Identification of ceRNA sets containing AR-lncRNAs involved in leaf senescence. **(A)** Framework used to predict the ceRNA sets containing AR-lncRNAs. **(B)** Alignment of representative miRNAs and AR-lncRNAs. **(C)** ceRNA regulatory networks. **(D)** Three representative network modules showing the identified leaf senescence-related ceRNA sets. **(E)** Enriched GOBPs of mRNAs involved in ceRNA sets.

the stabilization of *APG9* mRNA level *in trans* through RNA–RNA interaction, thereby playing a role as a negative regulator of leaf senescence.

AT2G14878-ADENINE PHOSPHORIBOSYL TRANSFERASE 1 (*APT1*) was predicted as a component of another regulatory module. APT1 inactivates cytokinin by catalyzing its conversion from free bases to nucleotides, and the loss-of-function *apt1* mutant exhibits delayed leaf senescence in darkness (Zhang et al., 2013). The results of interaction energy analysis and the co-expression pattern of *APT1* and *AT2G14878* raised the possibility that *AT2G14878* regulates dark-induced leaf senescence involving cytokinin metabolism (Figure 5C). The *at2g14878* knockout

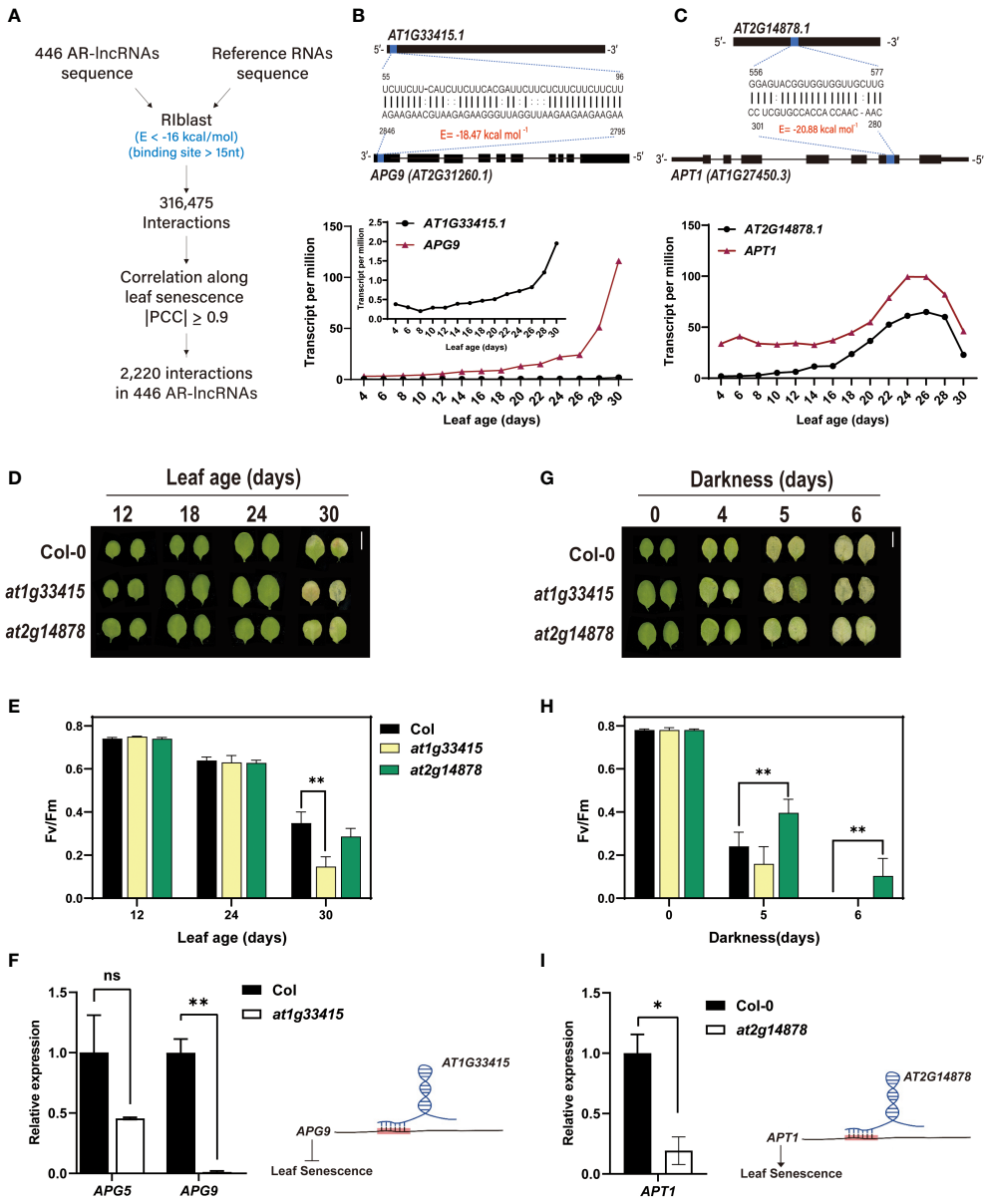

FIGURE 5
Prediction and validation of leaf senescence-regulating AR-lncRNAs. **(A)** Computational pipeline for the prediction of putative AR-lncRNA-RNA interactions. Interaction energy (**E**, kcal/mol) of AR-lncRNA-RNA pairs was calculated using RIBLAST 1.1.1. Expression correlation along leaf aging was used to reduce the number of AR-lncRNA-RNA pairs, resulting in the identification of 2,220 candidate pairs (|PCC| ≥ 0.9). **(B, C)** Interaction between *AT1G33415* and *APG9* **(B)** and between *AT2G14878* and *APT1* **(C)** (upper panel), and age-dependent expression patterns of *AT1G33415* and *APG9* **(B)** and *AT2G14878* and *APT1* **(C)** (lower panel). Expression levels were expressed as the mean TPM values of two biological replicates. **(D, E)** Leaf yellowing phenotype **(D)** and F_v/F_m ratio **(E)** of the third and fourth rosette leaves of *at1g33415* and *at2g14878* mutants during natural leaf senescence. Error bars represent standard deviation (SD; $n = 4$). Asterisks indicate significant differences between the indicated groups (t- test; **, $p < 0.01$). **(G, H)** **(G)** Leaf yellowing phenotype **(G)** and **(H)** F_v/F_m ratio **(H)** of *at1g33415* and *at2g14878* mutants subjected to dark-induced leaf senescence. Error bars represent SD ($n = 6$), and asterisks indicate significant differences between the indicated groups (t-test; **, $p < 0.01$). **(F)** Expression analysis of *APG5* and *APG9* in wild-type (Col-0) and *at1g33415* leaves by qRT-PCR (left), and *AT1G33415*-mediated suppression of leaf senescence potentially through interaction with *APG9* mRNA (right). **(I)** Expression analysis of *APT1* in wild-type (Col-0) and *at2g14878* leaves by qRT-PCR (left), and *AT2G14878*-mediated acceleration of leaf senescence potentially through the stabilization of *APT1* mRNA (right). In **(F)** and **(I)**, data in the left panel represent the mean of two replicates, and error bars represent SD ($n = 2$). Asterisks indicate significant differences (t-test; ns, non-significance; *, $p < 0.05$; **, $p < 0.01$).

mutant did not exhibit altered leaf senescence phenotype during aging (Figures 5D, E). However, similar to the extended longevity of *atp1* leaves under dark-induced leaf senescence, the *at2g14878* leaves showed a delayed senescence phenotype when leaf yellowing symptom was monitored in darkness (Figure 5G). The F_v/F_m of *at2g14878* leaves was also maintained for a longer duration during dark incubation, supporting the positive role of *AT2G14878* in dark-induced leaf senescence (Figure 5H). The senescence phenotype was further confirmed by examining expression of *SAG12* (Supplementary Figures 5A, B). To determine whether delayed senescence in the *at2g14878* mutant is caused by the suppression of *APT1* expression, the transcript level of *APT1* was analyzed in Col-0 and *at2g14878* leaves. To rule out the possibility that *APT1* expression was altered by delayed senescence, leaves at the early maturation stage were utilized for this experiment. As expected, the transcript level of *APT1* was significantly suppressed in the mutant (Figure 5I). This suggests that *AT2G14878* might promote cytokinin metabolism during leaf senescence by interacting with *APT1*, which decreases the cytokinin content of aged leaves.

Overall, the AR-lncRNA-mRNA pairs identified in this study provide valuable information for elucidating the biological function of lncRNAs, and will help to explore new lncRNA-mediated regulatory pathways involved in leaf senescence.

Discussion

Leaf development involves a series of functional and regulatory transitions from biogenesis to degeneration, which should be tightly regulated by coordinated molecular processes. Previously, we constructed a high-resolution and multidimensional transcriptome map to understand the fundamental transcriptional programs underlying age-dependent developmental shifts that occur during leaf development in *Arabidopsis*. Using these datasets, we performed comprehensive profiling of molecular processes active during leaf aging, and revealed coordinated transcriptional programs including transcriptional regulation by transcription factors and post-transcriptional regulation by various types of sRNAs (Woo et al., 2016).

Emerging evidence shows that lncRNAs play crucial roles in many biological processes and function through diverse mechanisms at multiple regulatory levels. However, the role of lncRNAs in leaf senescence regulation has not been investigated to date. Recently, a study in flag leaf senescence of rice reported the list of lncRNAs expressed during aging (Huang et al., 2021). Also, the study in the tomato analyzed lncRNA expression during the leaf senescence process (Li et al., 2022). However, the role of lncRNAs in *Arabidopsis* leaf senescence regulation has not been comprehensively investigated to date. In this study, we systematically identified 771 lncRNAs, including 539 annotated and 232 novel lncRNAs, during *Arabidopsis* leaf development. One of the challenges of lncRNA research in *Arabidopsis* is to explore the uncharacterized functions of lncRNAs. In general, bioinformatics tools predict certain transcripts as lncRNAs based on their sequence characteristics; however, these strategies are not suitable for inferring the function of lncRNAs. To overcome this problem, we employed several computational tools to infer the hidden functions of lncRNAs in leaf development and aging.

We integrated orthogonal sequencing datasets, such as ribo-seq data, to classify the 771 detected lncRNAs into three functional groups: sRNA precursors, canonical lncRNAs, and ribo-lncRNAs. In the ribo-lncRNA category, we identified novel lncRNAs that could potentially be translated into sORFs (sORF-encoding lncRNAs); however, experimental verification should be needed to determine whether these lncRNAs generate peptides/proteins as predicted, and under which conditions these peptides/proteins are actively expressed. To ascertain the potential functional contribution of small peptides to leaf development including senescence, it is necessary to examine the effect of mutations in the stop or start codons of the predicted small peptides. Some lncRNAs have also been reported to play dual roles; lncRNAs such as ENOD40 encode a small peptide as well as function as regulatory RNAs (Bardou et al., 2011). Other molecules embedded in lncRNAs are precursors of sRNAs such as miRNAs, which are processed from the introns of lncRNAs. These lncRNAs may not possess functions other than generating sRNAs; nonetheless, it is possible that processed lncRNAs act as modulators of target gene expression.

Of the 771 lncRNAs detected in leaves, 446 AR-lncRNAs were differentially expressed along aging. Intriguingly, the expression of AR-lncRNAs was regulated more dynamically in senescing leaves than in growing leaves (Figure 2A), revealing the contribution of these lncRNAs to leaf senescence. We also found that a large proportion of AR-lncRNAs (65.2%) was upregulated during leaf senescence (Figure 2D). Similarly, Huang et al. reported that the number of upregulated lncRNAs is higher than that of downregulated lncRNAs in late-senescence stage leaves compared to early booting stage leaves (FL1) in rice (Huang et al., 2021). Moreover, Li et al. observed a bigger number of upregulated genes than downregulated genes during tomato leaf senescence (Li et al., 2022). Such a characteristic expression pattern of AR-lncRNAs conserved in all three plant species might reflect that leaf senescence, despite its degenerative nature, involves a tightly-regulated program and has evolved to achieve biological processes that contribute to plants' fitness, such as nutrient relocation.

Integration of AR-lncRNAs with other transcriptome datasets generated under stress conditions revealed that AR-lncRNAs strongly overlap with lncRNAs potentially involved in stress responses (Supplementary Figure 3B). Overlapping AR-

lncRNAs might be involved in the protection of cellular integrity needed for the progression of leaf senescence, eventually leading to cell death. Our results, together with previous mRNA transcriptome data (Breeze et al., 2011; Woo et al., 2016), imply that leaf senescence is an intricate process, in which diverse environmental effects are superimposed on the age-dependent program. This mechanism would increase plant fitness in changing environments.

In this study, we used several different approaches to infer the biological function of AR-lncRNAs. Given that lncRNAs might regulate the expression of neighboring protein-coding genes *in cis* (Yang et al., 2013; Liu and Lim, 2018), the potential co-expression pattern of AR-lncRNAs and their cognate sense genes was first analyzed. Consistent with previous studies (Zhao et al., 2018), the expression of AR-lncRNAs with *cis*-NAT type was correlated with that of neighboring protein-coding genes. Notably, genes encoding proteins involved in cytokinin metabolic/catabolic processes were strongly enriched. Cytokinin is involved in cellular maintenance, suppressing senescence (Kim et al., 2006b). Thus, these AR-lncRNAs might be potential candidates that participate in leaf senescence by regulating cytokinin metabolism. We have also demonstrated that antisense AR-lncRNA *AT5G01595* might serve as a negative regulator in leaf senescence by modulating the expression of *FER1* that is an important player of iron-detoxification during leaf senescence.

ceRNAs play important roles in the regulation of biological processes; for example, IPS1 is involved in phosphate homeostasis. In the current study, we generated a list of developmental age-induced ceRNA networks in *Arabidopsis*, which will be useful to infer the physiological functions of AR-lncRNAs and their regulatory mode in the age-dependent program. *AT4G36648-miR164-ORE1* is a representative ceRNA network identified in this study that potentially regulates leaf senescence. ORE1 is one of the master transcriptional regulators of leaf senescence, and its expression must be elaborately regulated. *ORE1* is regulated at the transcriptional level by ETHYLENE INSENSITIVE 3 (EIN3), PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), and PIF5 (Sakuraba et al., 2014), and at the post-transcriptional level by *miRNA164* (Kim et al., 2009). Our results suggest the AR-lncRNA *AT4G3664* as another regulator of *ORE1* expression, although we have not yet experimentally validated this finding. Functional analysis of *AT4G3664* would provide mechanistic insights into how a robust regulatory network involving *ORE1* is organized and how it functions to modulate leaf senescence.

Given that interactions with regulatory RNAs are important for coordinating gene expression and regulating mRNA stability or splicing as well as translation of target genes through base-pairing interactions, we calculated the RNA–RNA interaction potential and also utilized the co-expression analysis approach to infer the putative functions of AR-lncRNAs. The AR-lncRNA-mRNA pairs identified in this study may serve as an initial resource for exploring the hidden regulatory pathways of leaf senescence.

As a proof of concept, two AR-lncRNAs were tested using the genetic approach. Loss-of-function mutations of two AR-lncRNAs resulted in altered senescence symptoms, demonstrating that these two AR-lncRNAs are essential for modulating leaf senescence. Low levels of target mRNAs in AR-lncRNA mutants as well as highly correlated gene expression patterns of paired AR-lncRNAs and mRNAs further support that both AR-lncRNAs regulate the stability of target mRNAs. It should be noted that the expression of target genes was analyzed during the early leaf maturation phase to rule out the possibility that reduced expression of target gene in the mutants was caused by the altered leaf senescence phenotype. Targets of these lncRNAs were identified as genes involved in autophagy as well as cytokinin metabolism, and both these processes are important for leaf senescence. To fully elucidate the detailed regulatory mechanism of how AR-lncRNAs affect the stability of target transcripts, further experiments such as RNA-pulldown assays (which would reveal direct RNA–RNA interactions) and genetic analysis of transgenic plants with mutated interaction sites need to be conducted.

Overall comparison of lncRNAs identified in *Arabidopsis*, rice and tomato in the context of leaf senescence revealed interesting features. In the case of rice, in total 3953 lncRNA were identified, which is composed of intergenic non-coding RNAs (lincRNAs) (2262, 57.2%), antisense lncRNAs (1260, 31.9%), sense lncRNAs (338, 8.55%) and intronic lncRNAs (93, 2.35%). In the case of tomato, in total 2074 lncRNAs were identified including intergenic lncRNAs (~55%), intronic lncRNAs (~25%), bidirectional lncRNAs (~10%), sense lncRNAs (~5%), and antisense lncRNAs (~5%). If we compare our result in *Arabidopsis* with those other species, all three species show a similar proportion of intergenic lncRNAs, which is the largest category among others. However, it is interesting that the proportion of antisense lncRNAs in tomato (~5%) is much smaller than that of *Arabidopsis* (39.4%) and rice (31.9%). This suggests the existence of different usage of lncRNA categories participating in the regulation of leaf senescence in different plant species. Huang et al. also constructed ceRNA network linking mRNA-miRNA-lncRNA for flag leaf of rice, as we reported ceRNAs in this study for *Arabidopsis*. Interestingly, ceRNA modules involving *miR164* were detected in both Huang et al. (rice) and our study (*Arabidopsis*), which revealed the evolutionary conservation of the *miR164* functionality in leaf senescence *via* forming ceRNA network.

In terms of functionality, our study revealed that cytokinin catabolic process, flavonoid glucuronidation, defense response, and oxidation-reduction process is enriched in genes overlapped with antisense AR-lncRNAs during *Arabidopsis* leaf aging. Additionally, putative target genes of ceRNA network are involved in the processes such as regulation of transcription, leaf senescence, regulation of auxin metabolic process, formation of plant organ boundary, lateral root development, and floral

organ senescence. Interestingly, enrichment of the oxidation-reduction process and regulation of transcription is commonly observed in the putative target genes of lncRNAs during rice leaf aging (Huang et al., 2021). Enrichment of oxidation-reduction related process in the putative target genes of lncRNAs is also conserved in tomato leaf senescence (Li et al., 2022). Huang et al. study additionally revealed that lipid metabolic process, transmembrane transport, and response to hormone processes are significantly enriched by the target genes of lncRNAs during rice leaf aging. In the case of leaf senescence in tomato, photosynthesis and starch and sucrose metabolism are additionally enriched by target genes of lncRNAs. These comparisons showed the existence of shared and distinct biological processes regulated by target genes of lncRNAs during leaf senescence in different plant species.

Based on the above-mentioned bioinformatics analyses, we not only systematically identified the lncRNAs over the leaf lifespan but also comprehensively reported the potential roles of these lncRNAs. Our predictions will open a new avenue for understanding *Arabidopsis* lncRNAs, by providing a comprehensive and confident list of lncRNA sets, and highly likely novel interactions between lncRNAs and mRNAs. Also in the future molecular studies, instead of focusing on one layer of molecules such as lncRNAs, mRNAs, or miRNAs individually, their network module needs to be experimentally investigated altogether, to study the emergent function of those networks which couldn't be uncovered by experiments focusing on the individual species.

Data availability statement

Publicly available datasets were reanalyzed in this study. The data used in the study are deposited in the Gene Expression Omnibus (GEO) repository, accession numbers are as follows: GSE42695, GSE120709, GSE43616, GSE69802.

Author contributions

JK, JuhL, MK, HJ, and PL designed research. JK, JuhL, MK, and JusL analyzed data. HL and TT performed research. JK, JuhL, MK, HJ, and PL wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1068163/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Coverage and RT-PCR data of *Arabidopsis* lncRNAs. **(A)** Gene view of the mRNA coverage of novel lncRNAs. **(B, C)** Validation of novel lncRNAs by RT-PCR analysis of total RNA.

SUPPLEMENTARY FIGURE 2
AR-lncRNAs mainly localize to the nucleus. **(A)** Heat map showing the relative abundances of AR-lncRNAs in the cytosolic and nuclear fractions of RNA samples prepared from 2-week-old seedlings. Rows are ordered based on hierarchical clustering. Color bar shows the fraction density between cytosol and nucleus. **(B)** Summary of sub-localization of lncRNAs and AR-lncRNAs. **(C)** Expression analysis of representative novel AR-lncRNAs in the cytosolic and nuclear fractions of RNA samples by qRT-PCR. Color of the AR-lncRNA names indicate the categories based on their localization as shown in **(B)** (pink: Nucleus only, orange: Nucleus > Cytosol, dark blue: Cytosol only, skyblue: Cytosol > Nucleus).

SUPPLEMENTARY FIGURE 3
AR-lncRNAs are differentially expressed under stress conditions. **(A)** Heat maps representing the differentially expressed lncRNAs (DE-lncRNAs) in *Arabidopsis* plants treated with ABA, drought, and cold ($p < 0.05$, |log2 (fold change) | $\geq$ 1). Rows are ordered based on hierarchical clustering. Color bar represents the gradient of log2(fold change) values compared with the before-treatment control. **(B)** Venn diagram indicating the numbers of lncRNAs showing differential expression in senescence, ABA, drought, and cold treatments.

SUPPLEMENTARY FIGURE 4
Genomic structure of *FER1* and *AT5G01595* and changes in senescence-associated gene expression during developmental leaf senescence. **(A)** Schematic structure of *FER1* and *AT5G01595*. The filled boxes for exons and the T-DNA insertion sites are indicated. **(B, C)** Senescence marker

genes expression in wild-type (Col-0) and *at5g01595*. (B) *SAG12* and **(C)** *CAB2* expressions were detected by qRT-PCR in third and fourth leaves along the leaf age.

SUPPLEMENTARY FIGURE 5

Changes in senescence-associated gene expression in *at1g33415 and at2g14878* during developmental and dark-induced leaf senescence. **(A, B)** Expression analysis of *SAG12* in wild-type (Col-0), *at1g33415* and *at2g14878* leaves during developmental **(A)** and dark-induced leaf senescence (B) by qRT-PCR. In **(A)** and **(B)**, data the mean of two replicates, and error bars represent SD (n = 2). Statistical analysis was performed using student's t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p<0.001$).

SUPPLEMENTARY DATA SHEET 2

Annotated lncRNAs.

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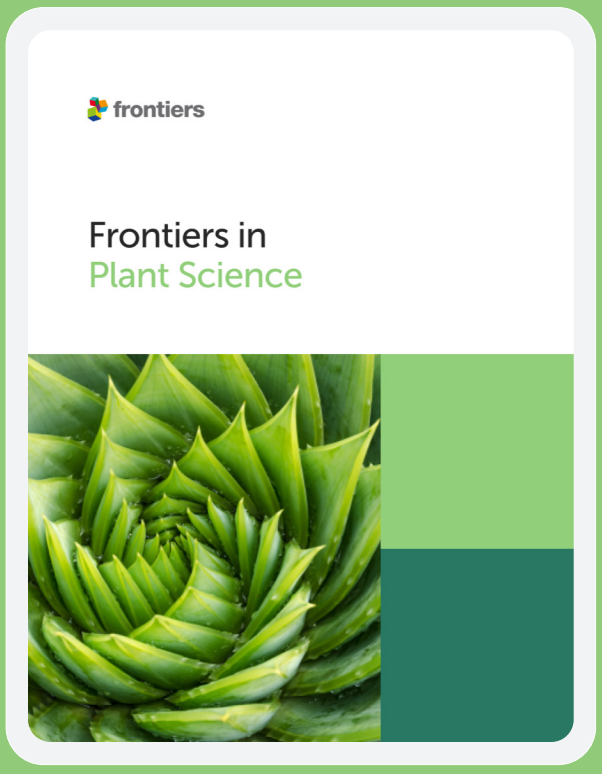

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