A MASTER'S THESIS

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

Doan Phan Phuong Thao

Department of Interdisciplinary Graduate Program in

Advanced Convergence Technology and Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

FEBRUARY, 2022

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

Doan Phan Phuong Thao

(Supervised by Professor Jeong-sik Kim)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science

2021. 12.

This thesis has been examined and approved.

Man Young Jung

Thesis director, Dr. Man-Young Jung, Prof. of Department of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science, Jeju National University

Hongshik OH

Jeongsik Kim

(Name and signature)

Date

Department of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

CONTENTS

ABBREVIATIONS

AmiRNA:		artificial micro RNA
DAB	:	3,3’-diaminobenzidine
GFP	:	Green fluorescent protein
GLK	:	Golden2-likes
LUC	:	Luciferase
NBT	:	Nitrotetrazolium blue chloride
ORE	:	ORESARA
PCR	:	Polymerase chain reaction
PEG	:	Polyethylene glycol
RAV1	:	Related to ABSCISIC ACID INSENSITIVE3 /VIVIPAROUS1 1
RLUC	:	Renilla luciferase
ROS	:	Reactive oxygen species
RPK1	:	Receptor-like protein kinase 1
SA	:	Salicylic acid
SAG12:		Senescence associated gene 12
SAGs	:	Senescence associated genes
SEN4	:	Senescence 4
TF	:	Transcription factor

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Senescence is the final stage of a plant's life history, however, it is necessary for plant succession as a beneficial developmental process. Senescence involves a series of programmed disassembly and degenerative events which are recycled to use in newly developing organs or offsprings. Plants, like other organisms, show two types of senescence: mitotic and post-mitotic senescence (Gan and Amasino, 1997; Guo and Gan, 2005). Mitotic senescence occurs in germ-like shoot apical meristem containing multipotent stem cells to produce organs, which is similar with replicative senescence or replicative aging in mammalian cells and yeast. Contrarily, post-mitotic senescence occurs in somatic cells of mature organs such as leaves and flowers. Post-mitotic senescence resembles degenerative senescence that occurs in somatic tissues of the animal adult body. Cells in these organs rarely undergo cell division but undergo cell growth, maturation, senescence, and eventual death. Leaf senescence is a type of post-mitotic senescence. Leaves undergo a series of developmental and physiological shifts during their lifespans (Woo et al., 2016). At the growth and maturation stages, leaves accumulate chemical energy and biomass through photosynthesis and other biogenesis processes involving cell division, differentiation, and expansion. When plants determine the fate of leaves to be senesced after a prolonged productive photosynthetic period, mature leaves undergo a reduction of photosynthetic activity, the major source of nitrogen and carbon, along with dismantlement of chloroplasts and degradation of chlorophyll, which results in color changes 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 in an orderly manner. Increased catabolic activities in senescing leaves are required for redistribution of nutrients and energy to other newly developing organs or seeds, which leads to the increased reproductive success in plants. For their efficient remobilization, various types of transporters for the molecules composed of carbon, nitrogen, and phosphate are activated. Furthermore, plant leaves sustain self-maintenance activities, such as pathogen defense and detoxification of reactive oxygen species to complete their redistribution activities during leaf senescence (Kim et al., 2018b). Leaf senescence is therefore an important and elaborately regulated phase in a plant's lifespan that critically contributes to the fitness of plants, ensuring better survival and optimal production of their offspring.

Plants evolve sophisticated genetic programs for determining the appropriate senescence onset and coordinating senescence progression. The onset and progress of leaf senescence require a range of certain developmental ages but are also triggered by the various external and internal factors. Internal factors influencing leaf senescence include age, hormones, ROS, and developmental processes such as reproductive growth. Leaf senescence can also occur prematurely by external factors such as extreme temperature, drought, salinity, nutrient deficiency, darkness, and pathogen infection (Figure 1) (Guo and Gan, 2005). For example, dark treatment has been widely used to induce uniform and rapid senescence as a typical senescence inducing condition in senescence assay (Wang et al., 2012). The initiation of leaf senescence is determined through the coordinated

actions of multiple 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). In addition, ORE1 regulates a subset of processes including the degradation of chloroplast during leaf senescence by interacting with GLK1 and GLK2, the G2-like transcription factors which are important for chloroplast development and maintenance. During early leaf development, GLKs are highly expressed to activate various downstream genes such as photosynthesis-related genes. As leaves get older, expression of *ORE1* is increased, which in turn, sequesters GLKs and reduces their transcriptional activity, shifting the balance from chloroplast maintenance towards deterioration (Rauf et al., 2013). Additionally, reactive oxygen species (ROS) signaling has also been suggested as a possible mechanism through which senescence is triggered (Munne-Bosch and Alegre, 2002). Previous studies demonstrated 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). On the other hand, the *ORE7/ESC* gene which encodes an AT-hook DNA-binding protein is involved in regulating chromatin organization at interphase and affect initiation of leaf senescence (Lim et al., 2007).

Leaf senescence is an active and acquired genetic process and is regulated by highly coordinated networks that induce an extensive reprogramming of gene expression. The

dynamic activation of senescence regulatory genes is thought to be a key mechanism that induces changes in expression of diverse executive senescence-associated genes (SAGs) for systemic biochemical and physiological processes during the progression of leaf senescence. For examples, *SEN4* and *SAG12*, encode xyloglucan endotransglucosylase/ hydrolase 24 and cysteine protease, respectively, which are mainly involved in macromolecule degradation, and are upregulated SAGs 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 regulated coordinately by time-dependent interaction among various positive and negative senescence regulatory elements including receptors, kinases/phosphatases, transcription factors (TFs), and epigenetic regulators. Among these, the TFs, such as NACs (NAM/ATAF/CUC) and WRKYs are important for regulating the temporal expression of senescence-associated genes during leaf aging. Time-evolving NAC networks undergo a temporal transition of regulatory interaction among NACs from the presenescent to the senescing stage and guide the timely induction of senescence processes, such as SA and ROS responses (Kim et al., 2018a).

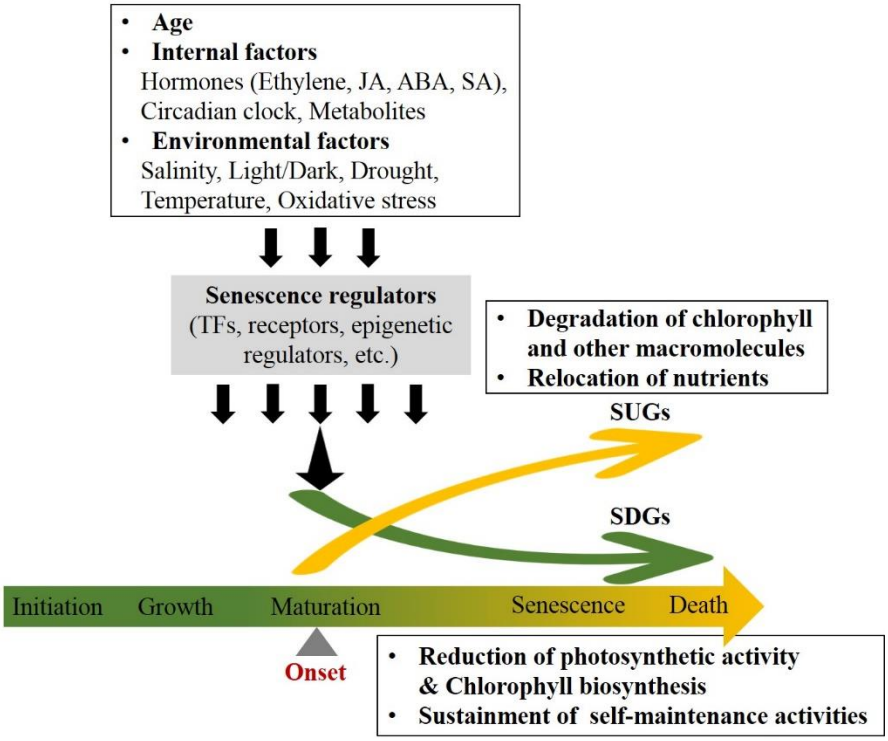

Figure 1. A hypothetical model for genetic pathways of leaf senescence (modified from Ali et al., 2018**)**. Leaf senescence occurs in an age-dependent manner, but the initiation and progression of senescence can be controlled by a variety of environmental factors such as salinity, light/dark, drought, temperature, and oxidative stress. It is also known that internal factors including several phytohormones, circadian clock, and metabolites also influence senescence. Coordinated actions of multiple senescence regulators are activated to determine the proper timing of senescence onset by recognizing and processing this internal and external information. During early leaf development (encompassing leaf initiation, growth, and maturation), leaves ensure efficient photosynthesis by regulating the biochemical processes including activation and suppression of chlorophyll biosynthesis and breakdown, respectively. When leaves enter senescence phase, the gradual breakdown of chlorophyll and subsequent catabolism of macromolecules occurs for redistribution of nutrients and energy to other newly developing organs or seeds. All of these substantial changes are associated with alterations in gene expression. Many genes expressed in mature leaves, such as the genes encoding photosynthetic enzymes, are downregulated upon the onset of senescence (SDGs). In contrast, many senescence-upregulated genes (SUGs) are transcriptionally upregulated during leaf senescence. Their dynamic activation by senescence regulatory genes is thought to be a key mechanism that induces or reduces changes in expression of diverse executive SDGs and SUGs for systemic biochemical and physiological processes during the progression of leaf senescence.

II. MAIN RESEARCH

1. SUMMARY

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 expression of a luciferase-based reporter that was activated by *SEN4* and *SAG12* promoters, early and late senescence-responsive genes, and effectors of identified 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 temporal 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.

2. BACKGROUND

Leaf senescence is a complex but highly regulated developmental process involving a coordinated sequence of multiple molecular events, which eventually leads to death of the leaf. The ultimate goal of leaf senescence research is to understand the composition, organization, and function of complex gene-regulatory networks that govern leaf senescence. With the completion of genomic sequencing of model organisms and the advent of omics technologies, substantial progress over the several past decades has improved our knowledge of the molecular mechanisms underlying leaf senescence. These technologies are highly valuable for identification of various types of key regulatory genes and their inferred gene regulatory networks responses to certain or multiple environmental or genetic inputs. Researches for leaf senescence have been largely relied on phenotypic evaluation of senescence responses in over-expression and mutant lines. However, with the advance of high-throughput approaches, more and more genetic information has been updated on public databases over the last few years. Therefore, a more rapid and efficient method to dissect the functional roles of genes is requisite. Currently, the temporal and temporal 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 that response to plant hormones or other environmental factors as well as many other molecular characterizations, such as protein-protein interaction, subcellular localization, and protein-DNA interaction (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 temporal 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, temporal 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.

3. MATERIALS AND METHODS

3.1. 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 ℃ (16 h light/8 h dark cycle; cool white fluorescent bulb with 100 to 150 μmol·m^2·sec^{-1}; TLD/840RS; Philips).

3.2. Plasmid Construction

We generated plasmid constructs for transient gene expression in protoplasts or tobacco using GATEWAY cloning technology (Invitrogen, USA). For the reporter plasmids (*SEN4-*, *SAG12-*, *CA1-*, *THIONIN-LUC*), the 5' upstream regions encompassing the *SEN4* (At4g30270), *SAG12* (At5g45890), *CA1* (At3g01500), *THIONIN* (At1g66100), and *PRK* (At1g32060) promoters were amplified by polymerase chain reaction (PCR) with Pfu DNA polymerase, using Arabidopsis Col-0 genomic DNA as a template and the following sets of primers (Table 1). We subcloned the amplified DNA fragments into the entry vector of pCR-CCD-R or pCR-CCD-F 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 (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 vectors, ORE1, ORE4, and ORE9 amiRNA plasmids were generated by digesting pAmiR-ORE1 (CSHL_075023), pAmiR-ORE4 (CSHL_027820), and pAmiR-ORE9 (CSHL_038083) 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 (http://wmd3.weigelworld.org) 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 (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. For miR164B-OX effector, the full-length expression cassette of miR164B was amplified using PCR from Arabidopsis Col-0 genomic DNA with Pfu DNA polymerase and gene-specific primers (Table 1). Further, we subcloned the amplified

DNA fragment into the entry vector of pCR-CCD-F using the corresponding restriction enzymes to produce entry clone. Then, we recombined the entry clone using gateway version of pCsVMV-N-999 to produce the effector plasmid of miR164B-OX.

3.3. Protoplast Isolation and Transfection

We isolated protoplasts 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], 400mM mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES-KOH [pH 5.7], and 0.1% BSA [Sigma A6793, USA]) 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 x 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. Then, we prepared effector plasmids of overexpression or amiRNA and reporter plasmids by CsCl gradient purification using an ultracentrifuge (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, 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, USA) 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.

3.4. Luminescence Measurement

We analyzed the expression of the specific senescence reporters by temporal measurement of luciferase activity in protoplasts. Further, 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, USA) of a white and round bottom 96-well microplate. The microplate was covered with a clear plastic cap and incubated at 22 ℃ in the dark on a GloMax 96 microplate luminometer (Promega, USA). For three 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. Normalized LUC expression was calculated using the formula:

$$\textbf{Normalized LUC expression} = \frac{\textbf{LUC luminescence}}{\textbf{RLUC luminescence}} * \textbf{100}$$

3.5. RNA Extraction and quantitative Reverse Transcriptase-PCR (qRT-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, USA). Further, qPCR was performed on a CFX96 real-time qPCR detection system (Bio-Rad, USA) using appropriate primer sets. We designed primers for candidate genes using Primer 3 software (Untergasser et al., 2012) and are listed (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.

3.6. 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 ℃ 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, USA) and nitrotetrazolium blue chloride (NBT; Sigma N6639, USA) were used, respectively. Tobacco leaves transiently overexpressing *ORE1*, *RPK1*, and control were

subjected to DAB and NBT staining at the indicated days. The leaves were soaked in 1 $mg \cdot mL^{-1}$ DAB or 2 $mg \cdot mL^{-1}$ 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 $mg \cdot mL^{-1}$ trypan blue 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.

3.7. Protoplast Viability Test and Subcellular Localization

Cell death of Arabidopsis protoplasts was assayed by Evans blue staining (Sigma E2129, USA). The protoplasts overexpressing *ORE1*, *RAV1*, *RPK1*, *ORE7*, and control *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 protoplast cells using Zeiss Axiostar Plus Microscope (Ambastha et al., 2017). Stained protoplasts were counted in fives 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%.

We determined the subcellular localization of control GFP, ORE1, RAV1, RPK1, and ORE7 in protoplasts 18 h after transfection using an epifluorescence microscope (Axioscope A1, Carl Zeiss, Germany) and a BP505-530 filter. We processed and pseudocolored images using Photo Pos Pro 3 (Power of Software) or Photoshop 2018 (Adobe Systems).

Table 1. The lists of primers used in this study.

Plasmid construction

Effecter

Primer Name	Sequences (5' to 3')[1)]	R.E.[2)]	Vector
ORE1-F	CAAACTAGTATGGATTACGAGGCATCAAGAATC	*Spe*I	pCR-CCD-F
ORE1-R	CAAAGGCCTGAAATTCCAAACGCAATCCAATTC	*Stu*I	
RAV1-F	TTTGGATCCGGATGGAATCGAGTAGCGTTGAT	*Bam*HI	pCR-CCD-F
RAV1-R	TTTAGGCCTCGAGGCGTGAAAGATGCGTTGCT	*Stu*I	
RPK1-F	ATTCTGCAGATGAAACTTCTGGGTTTGGTCTTCTT	*Pst*I	pCR-CCD-F
RPK1-R	ATTAAGCCTACAATCTAGAAGGCTGGATTCGTTT	*Hind*III	
ORE7-F	GGACTAGTATGGAAGGCGGTTACGAGCA	*Spe*I	pCR-CCD-F
ORE7-R	GAAGGCCTAAAAGGTGGTCTTGAAGGTGT	*Stu*I	
miR164B-F	TCTCTGCAGTTTTTGGGTAGCATGTTCAT	*Pst*I	pCsVMV-N-999
miR164B-R	TCTAGGCCTCGCTAACCGAAACTATGTTC	*Stu*I	
CsVMV AmiR-	GGTGTAAGCTATTTTCTTTGAAGTAC		pCsVMV-PP2C-AmiR
AmiR nos-R	GCAACAGGATTCAATCTTAAGA		
RPK1 AmiR-I	TATAAGTCGAACCGAAACCAC		pCsVMV-PP2C-AmiR[a)]
RPK1 AmiR-II	GTGGTTTCGGTTCGACTTATA		pCsVMV-PP2C-AmiR[b)]
RPK1 AmiR-III	GTAGTTTCGGTTCCACTTATT		pCsVMV-PP2C-AmiR[c)]
RPK1 AmiR-IV	AATAAGTGGAACCGAAACTAC		a) + b) + c)

Reporter

Primer Name	Sequences (5’ to 3’)[1)]	R.E.[2)]	Vector
SAG12pro-F	TTTGAGCTCGTTGGTACTTTGGTAGCAAGTCG	*Sac*I	pCR-CCD-R
SAG12pro-R	TTTGGATCCTGTTTTAGGAAAGTTAAATGACTTTTG	*Bam*HI	
SEN4pro-F	TTTGAGCTCATTTGGGTTCGTATTCTTTCTCATAC	*Sac*I	pCR-CCD-R
SEN4pro-R	TTTGGATCCTGTCTTTGTGTGTGTGCGTACG	*Bam*HI	
THIONINpro-F	TTTGGATCCATTGAAAAACAGAGGGAGTATTGA	*Bam*HI	pCR-CCD-F
THIONINpro-R	TTTAGGCCTCTTTTGATTGATTAGTTGTTTGATCAC	*Stu*I	
CA1pro-F	TTTGGATCCCCATTCCTCGAGGTACTTAAC	*Bam*HI	pCR-CCD-F
CA1pro-R	TTTAGGCCTTTGTTGGCGAAGAGAAGCGGAGA	*Stu*I	
PRKpro-F	TTTGGATCCCCCTTACGATATAAGGTCTGA	*Bam*HI	pCR-CCD-F
PRKpro-R	TTTAGGCCTTGTTGTTTGTTTGGTGTTTTGGTC	*Stu*I	

1) Annealing temperature for all primers is 56℃ or 55℃.

2) R.E., restriction enzyme sites.

Real time PCR

Primer Name	Sequences (5’ to 3’)	Annealing temperature
ORE1-qPCR-F	CGTTTAGAAGGCAAATATTGTATTGA	60℃
ORE1-qPCR-R	AACACGACATATAACCCATTCGT	
RPK1-qPCR-F	TGTCTCCAACGAATGTGTTTGC	60℃
RPK1-qPCR-R	GCTCGCATGGTAACCGATTAAC	
ACT2-qPCR-F	CAGTGTCTGGATCGGAGGAT	60℃
ACT2-qPCR-R	TGAACAATCGATGGACCTGA	

Table 2. Calculated interaction likelihoods between amiRNAs and potential target gene(s).

amiRNA	amiRNA core sequence (5' → 3')	amiRNA source[a]	Potential target gene(s)	Target recognition sequence (5' → 3') [b]	Hybridization energy (kcal/mol) [c]	Perfect-match-dG cutoff (70%)[d]
ORE1	TTAACGAAGGT AAGCCGGTG	A	ORE1	CAGCCGGTTTA CCTTCGTTAA	-40.86	93.76
RPK1	TATAAGTCGAA CCGAAACCAC	W3	RPK1	GCGGTTTCGGT TCGACTTATA	-38.13	97.89
ORE4	TTAGTCTTACTG ACGGGTCTG	A	ORE4	AGACCCATCAG TAAGACTAAA	-36.51	85.34
ORE9	TCTTAGGGGAA CTGGTACCTA	A	ORE9	TAGCTACCAGT TCCCCTAAGT	-37.23	81.29

[a] A, From ABRC; W3, designed using the WMD3 (web microRNA designer3).

[b] Matched sequences between amiRNA and target sequence are underlined.

[c] Hybridization of amiRNA to its mRNA target site.

[d] Ratio of the hybridization energy between amiRNA and its target to that between amiRNA and its perfect reverse complement in percent.

4. RESULTS

4.1. Establishment of Luciferase-based Reporters Controlled by the Promoter of SAGs for Cell-based Senescence Assay

Arabidopsis protoplasts is 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 2, 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 (Woo et al., 2001; Woo et al., 2002). We also tested other *LUC* reporters driven by the promoters of *PRK*-, *CA1*-, and *THIONIN-LUC* with down-regulated 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 (Woo et al., 2016; Kim et al., 2018c). From these results, we conducted subsequent analyses using only the *SEN4-LUC* and *SAG12-LUC* reporters.

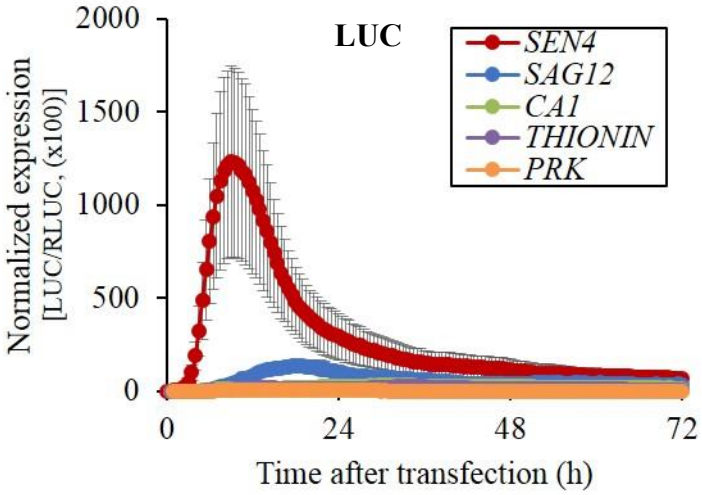

Figure 2. Bioluminescence expression patterns of luciferase reporters controlled by various senescence-associated promoters in Arabidopsis protoplasts during darkness. Bioluminescence traces in Arabidopsis protoplast cells expressing a firefly luciferase gene (*LUC*) controlled by senescence-associated genes (*SEN4*-, *SAG12*-, *CA1*-, *THIONIN*-, and *PRK*-*LUC*) and a Renilla luciferase (RLUC) controlled by 35S promoter (35S-RLUC) in darkness after transfection. Image acquisition was performed every 30 min for 3 d. 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.

4.2. 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 (Figure 3) (Lim et al., 2007; Kim et al., 2009; Woo et al., 2010; Koo et al., 2017).

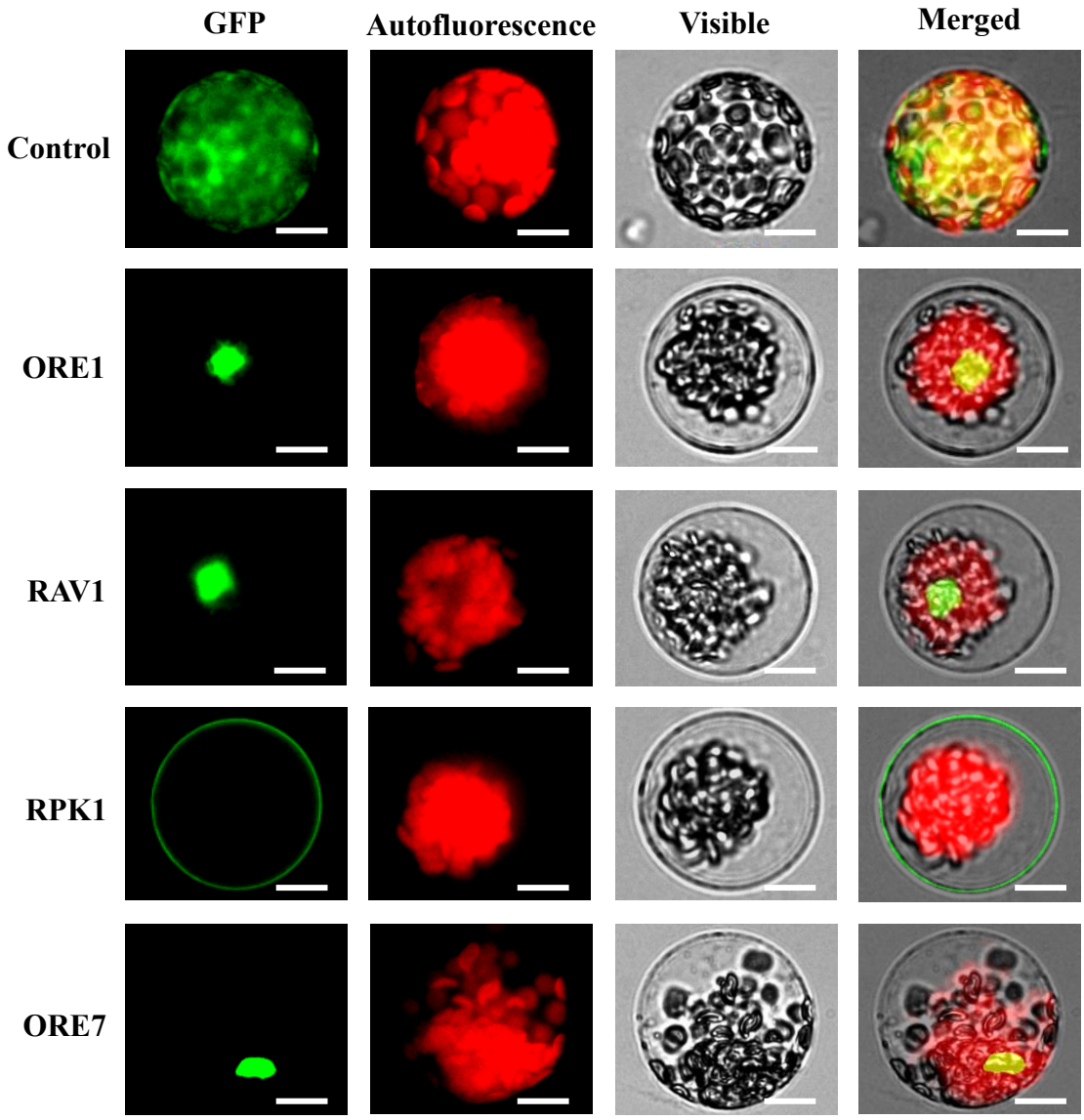

Figure 3. Subcellular localization of ORE1, RAV1, RPK1, and ORE7 in transiently transfected Arabidopsis protoplasts. ORE1, RAV1, RPK1, ORE7, and Control GFP were transiently expressed in Arabidopsis protoplast for 24 h under dim white light. GFP and Chloroplast autofluorescence (red) signals were monitored by fluorescent microscopy. GFP, autofluorescence, bright-field, and the merge images are shown. Bars = 100 μm.

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 4). 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 14- and 11-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 (Figure 4A and B). Interestingly, ectopic expression of *ORE1* induced distinct expression patterns of *SEN4*- and *SAG12-LUC* in that their expression was induced earlier but with higher-level at their peak time, compared with that of control, and was dampened quickly (Figure 4C, D, G, H). 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 (Figure 4E, F, G, H). 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 24 to 72 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., 2007; 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.

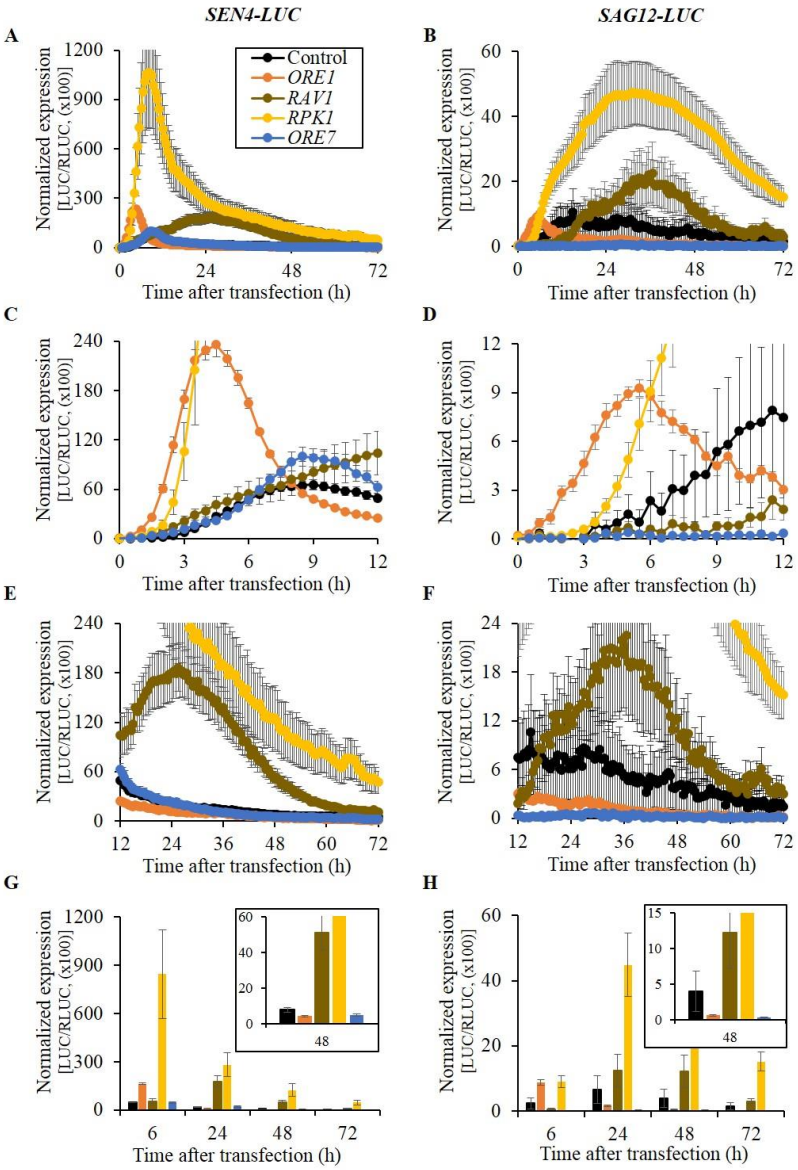

Figure 4. Expression patterns of *SEN4*- (A) and *SAG12-LUC* (B) reporters by overexpression of senescence regulators. Arabidopsis protoplast cells were co-transfected, as described in Figure 1, but with overexpression effectors of *ORE1*, *RAV1*, *RPK1*, and *ORE7* and either reporter of *SEN4*- or *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 hour; **C**, **D**) and late (12–72 hour; **E**, **F**) time points are shown. (**G**, **H**) Normalized expression of *SEN4*- and *SAG12-LUC* at 6, 24, 48, and 72 h after transfection, and inserted image with an adjusted scale at 48 h after transfection are shown. Data represent mean ± SE (n = 3). Similar results were obtained in two independent trials.

4.3. 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 (Figure 5A and 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., 2007; 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 2.5- and 2-fold reduction of *SAG12-LUC* expression at its peak time compared with control, respectively, although no effect on *SEN4-LUC* expression was observed (Figure 5C and D). 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 4), the amiRNAs approaches in protoplasts can be useful for assessing gene functions in senescence regulation.

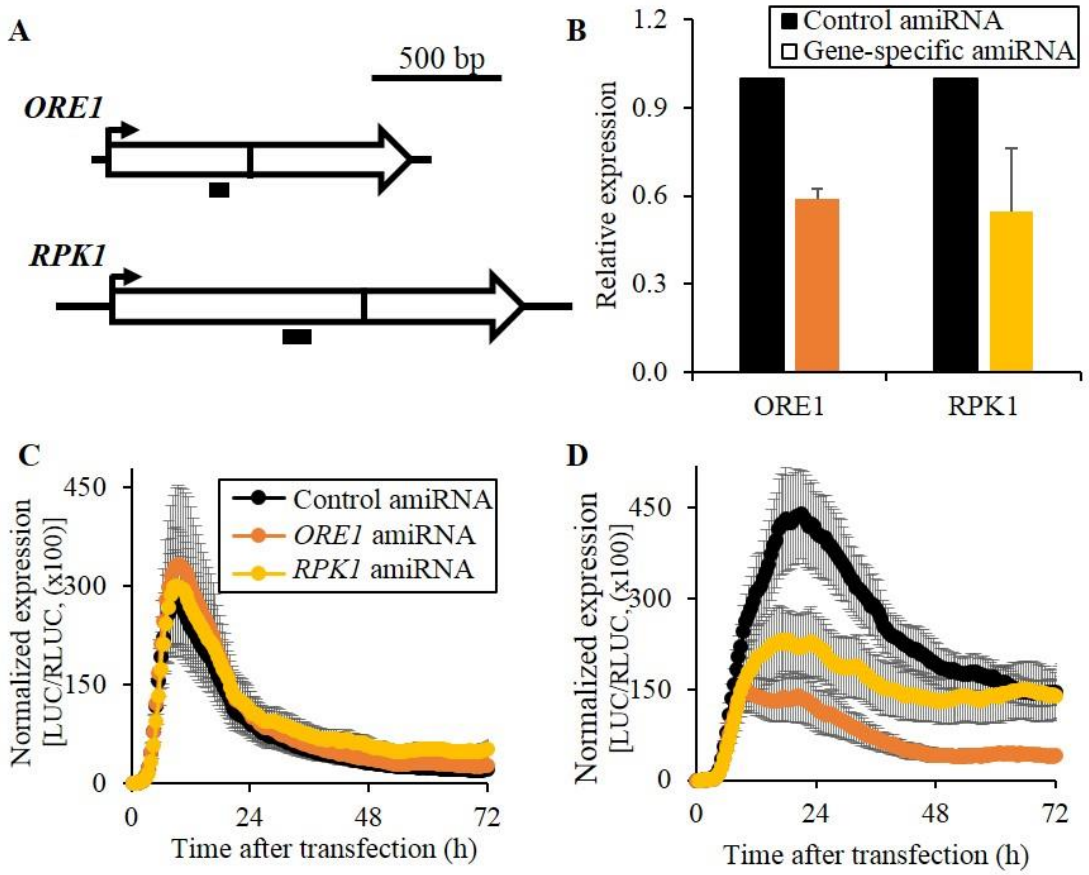

Figure 5. Expression patterns of *SEN4*- (A) and *SAG12-LUC* (B) reporters by reduced expression of senescence regulators using amiRNAs. (**A**) Schematic of the *ORE1* and *RPK1* with the amiRNA-targeted site (arrowheads) and the PCR amplified region (horizontal black bars) indicated for each. (**B**) 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** and **D**). Bioluminescence traces of *SEN4*- (**C**) and *SAG12-LUC* (**D**) with amiR-construct gene targeting *ORE1* or *RPK1* during darkness. Symbols in (**D**) are the same as in (**C**). Data represent mean ± SE (n = 3). Similar results were obtained in two independent trials.

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; Woo et al., 2002; Kim et al., 2009). We failed to detect any significant change in *SEN4-LUC* when *ORE9*, *ORE4* miRNAs, and miR164B-OX were introduced (Figure 6A), 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 (Figure 6B). 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* miRNAs 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.

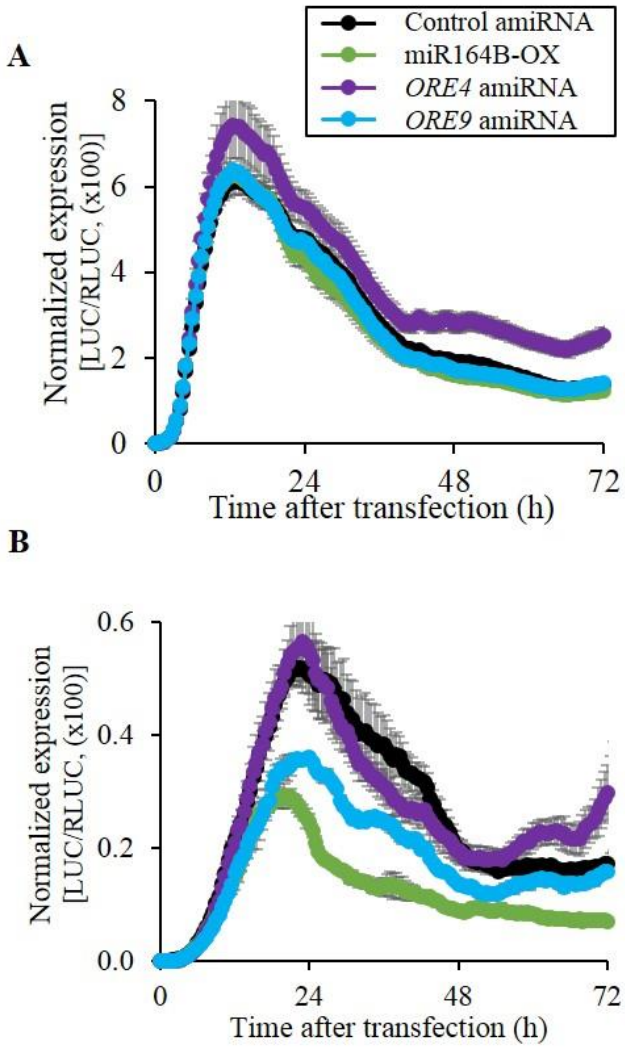

Figure 6. Bioluminescence traces of *SEN4*- (A) and *SAG12-LUC* (B) in other amiRNAs-transfected protoplast cells. Transfection and imaging acquisition was performed, as in Figure 3, but with a plasmid of miR164B-OX, *ORE4* amiRNA, and *ORE9* amiRNA as effectors. Data represent mean ± SE (n = 3). Similar results were obtained in two independent trials.

4.4. Divergent Function of ORE1 and RPK1 in Premature Cell Death Regulation through Different ROS Signaling

Overexpression of selected senescence regulators led to different temporal expression patterns of senescence reporters (Figure 4), with some of them, such as *ORE1* and *ORE7* inducing dampened expression. We explored cell death responses in protoplasts where ORE1, RAV1, RPK1, and ORE7 were overexpressed because senescence accompanies death, and suppressed expression could be induced by cell death. 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. 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 7). 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 temporal functions in triggering cell death during senescence.

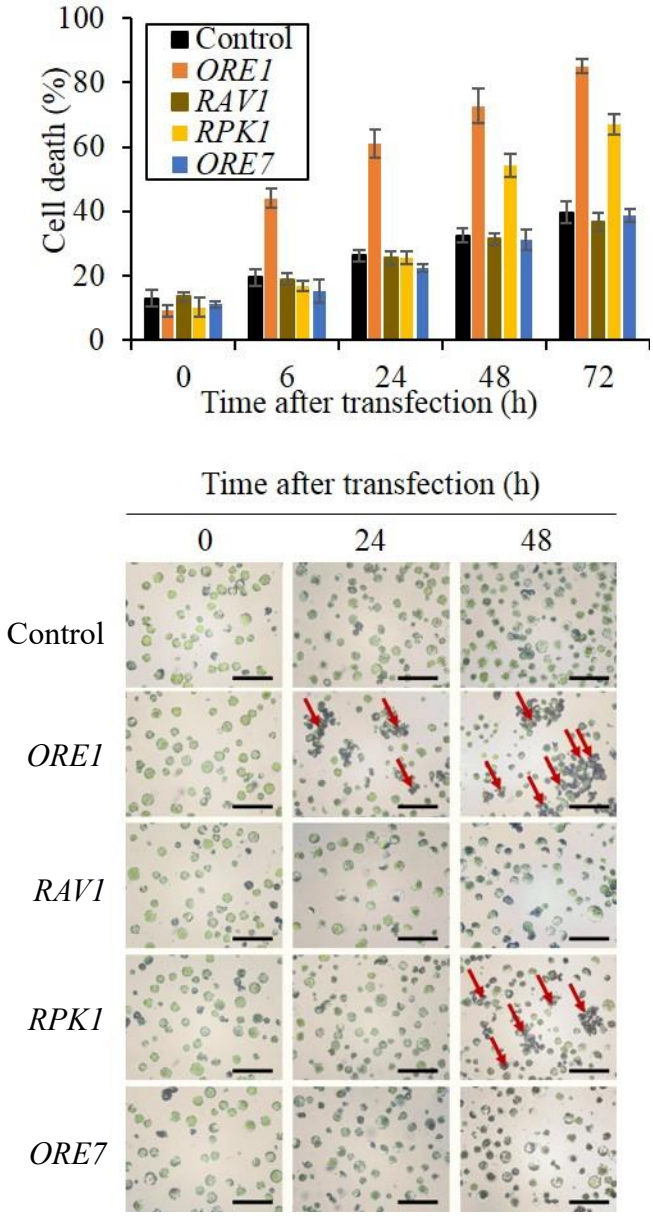

Figure 7. Temporal cell death responses induced by overexpression of various senescence regulators in Arabidopsis protoplasts. (**A**) Time-series measurement of cell death in protoplasts expressing various senescence regulators. (**B**) Representative images of protoplasts stained by Evans blue. Protoplasts were transfected with empty *GFP* (Control), *ORE1*, *RAV1*, *RPK1*, or *ORE7*, and stained with Evans blue dye at 6, 24, 48, and 72 h post-transfection. Normal and stained cells were counted in five to twelve 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 = 5–12). Similar results were obtained in two independent trials.

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 8). 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 *RPK1* and *GFP* control induced detectable cell death at 2 DAI and 3 DAI, respectively (Figure 8A). 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 overexpressing leaves (Figure 8B). 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 8C). 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.

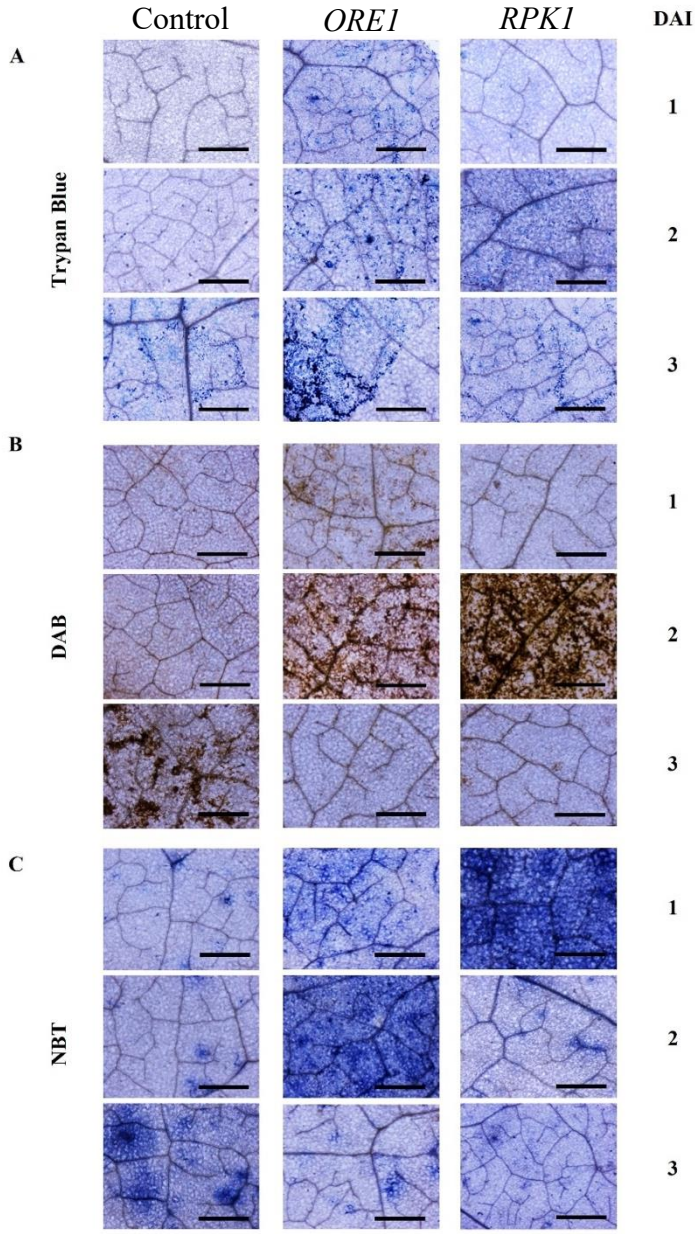

Figure 8. ROS accumulation and cell death responses in *N. benthamiana* leaves expressing various senescence regulators. *GFP* (Control), *ORE1*, or *RPK1* was transiently overexpressed in four weeks old *N. benthamiana* leaves through Agrobacteria infiltration. (**A**–**C**) trypan blue (**A**), NBT (**B**), and DAB (**C**) staining was performed using their leaves at the indicated days post infiltration (DAI). Bars = 100 mm. Similar results were obtained in three independent trials.

5. 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 temporal senescence response assay and validated the feasibility of functional assessment of genes in senescence regulation using their overexpression and knockdown approaches (Figures 4, 5, and 6). Furthermore, we investigated the functional difference between *ORE1* and *RPK1* in cell death-mediated senescence by combining the temporal responses of the reporter assay and histochemical assay (Figures 7 and 8).

5.1. 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 temporal responsiveness (Figure 2). *SEN4* and *SAG12* were identified as transcriptionally upregulated SAGs with different temporal 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. Also, we tested the possible usage of other down-regulated SAGs as potential reporters, but their expression levels were marginal or variable in the protoplasts (Figure 2). 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 using intact leaves (Figure 4) (Lim et al., 2007; Kim et al., 2009; Woo et al., 2010; Lee et al., 2011). Furthermore, knockdown approaches based on miRNA 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 with *SAG12-LUC* resulted in reduced *SAG12* expression in the protoplasts (Figure 5 and 6). 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 might be because transfected *SEN4-LUC* induces a certain level of expression before substantial removal of endogenous target genes by transfected amiRNAs. 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 (Figure 4C and D). This is consistent with the results using amiRNA approaches for *ORE1* and *RPK1* (Figure 5D). 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 temporal 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*) (Figure 4A and B). It is consistent with a previous report that AT-hook protein ORE7 functions as an epigenetic regulator for leaf senescence (Lim et al., 2007). 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 (Figure 4A and B). This implied that *ORE1* has a different molecular function in the regulation of senescence from the *ORE7*-mediated repression of SAGs.

5.2. 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 7). 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 4). 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 is likely involved in *ORE1* and *RPK1*-mediated cell death and senescence (Figure 8). 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 are 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.

5.3. Senescence Regulatory Scheme

Based on our study, we suggest a regulatory and temporal 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 (Figure 9). *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 temporal 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.

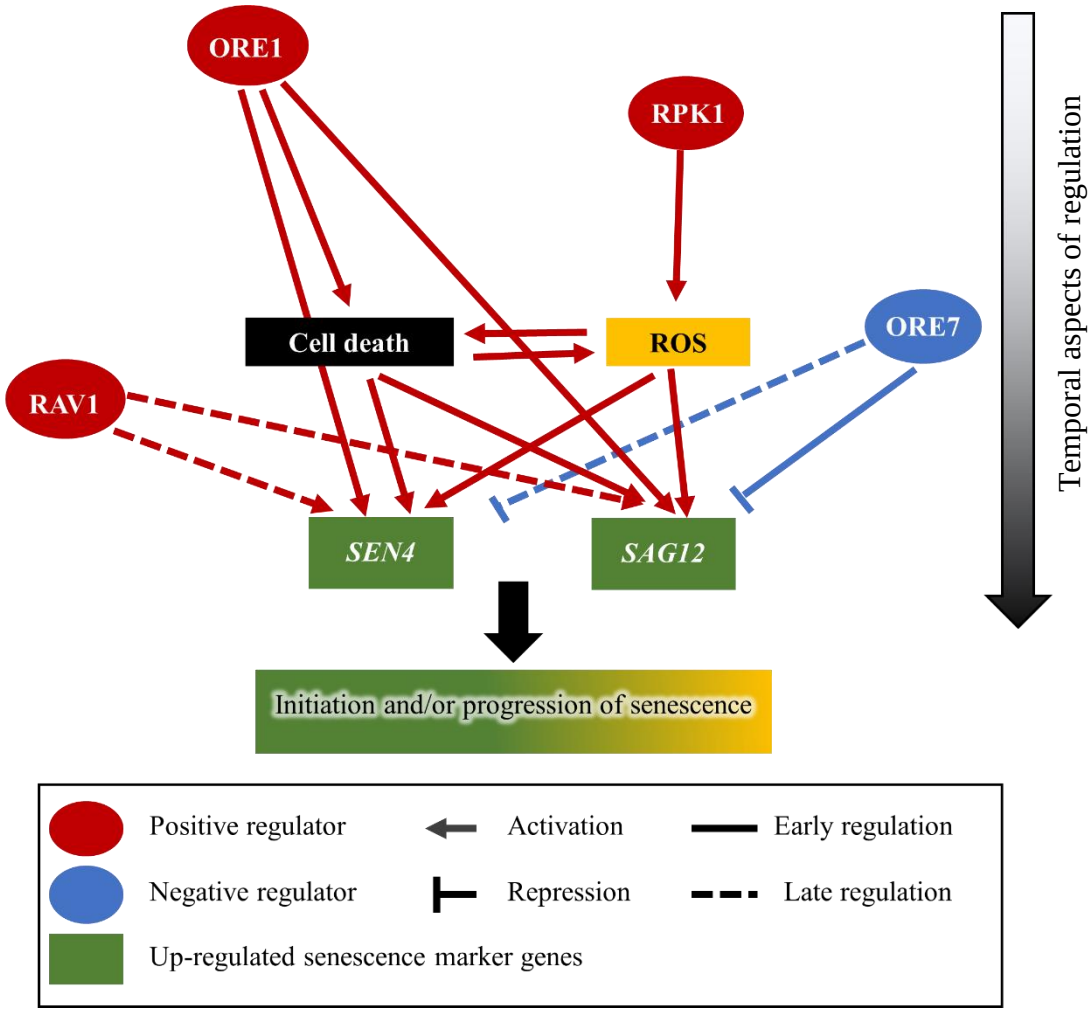

Figure 9. A regulatory scheme of the cellular senescence program. In this scheme, ORE1 and RPK1 trigger senescence regulatory programs in an early senescence stage through a different mechanism; ORE1 and RPK1 mediate cell death- and ROS-induced senescence, respectively. However, RAV1 is involved in late senescence responses separately from ORE1 and RPK1. ORE7 functions as a negative senescence regulator to repress the activity of *SAG12* promoter both in early and late stage, but that of *SEN4* promoter only in the late stage.

III. CONCLUSION

In this study, we established a rapid and systematic cellular approach to exploit senescence reporters with a luciferase under the control of senescence-associated promoter for a temporal analysis using protoplasts isolated from Arabidopsis leaves. We validated the feasibility of this system with known positive and negative senescence regulators through overexpression and amiRNA-based knockdown approaches. The time-series luminescence analysis of senescence reporters showed diverse temporal and expression patterns in response to altered expression of senescence regulators, indicating their differential temporal involvement in the regulation of leaf senescence. Combining histochemical assay for detecting ROS and cell death, we suggested a novel regulatory mechanism of *ORE1* and *RPK1* that mediate cell death processes through different ROS intermediate signals. Based on our works, we suggest a regulatory and temporal scheme of cellular senescence program regarding how senescence regulatory genes such as *ORE1*, *RPK1*, *RAV1*, and *ORE7* are involved in the regulation of senescence, reflected by the expression of *SEN4* and *SAG12*, partially through cell death and ROS-mediated signalling. Overall, we confirmed that the protoplast transient expression system based on the luciferase-based assay is a useful tool for rapid functional dissection of senescence regulators in Arabidopsis. Combining other cellular reporters or different sources of protoplasts will be able to extend the utility our approaches for studying various senescence processes in Arabidopsis as well as other non-model plants in which transgenesis is difficult, but protoplast system is facile.

IV. REFERENCES

Ali, A., Gao, X., and Guo, Y. (2018). Initiation, progression, and genetic manipulation of leaf senescence. *Methods Mol Biol.* 1744**,** 9-31. doi: 10.1007/978-1-4939-7672-0_2.

Ambastha, V., Sopory, S.K., Tiwari, B.S., and Tripathy, B.C. (2017). Photo-modulation of programmed cell death in rice leaves triggered by salinity. *Apoptosis.* 22(1)**,** 41-56. doi: 10.1007/s10495-016-1305-7.

Balazadeh, S., Wu, A., and Mueller-Roeber, B. (2010). Salt-triggered expression of the ANAC092-dependent senescence regulon in Arabidopsis thaliana. *Plant Signal Behav.* 5(6)**,** 733-735. doi: 10.4161/psb.5.6.11694.

Camargo Rodriguez, A.V. (2021). Integrative modelling of gene expression and digital phenotypes to describe senescence in wheat. *Genes (Basel).* 12(6). doi: 10.3390/genes12060909.

Domozych, D.S., Ritter, E., Lietz, A., Tinaz, B., and Raimundo, S.C. (2020). Protoplast isolation and manipulation in the unicellular model plant Penium margaritaceum. *Methods Mol Biol.* 2149**,** 111-124. doi: 10.1007/978-1-0716-0621-6_7.

Gan, S., and Amasino, R.M. (1997). Making sense of senescence (Molecular genetic regulation and manipulation of leaf senescence). *Plant Physiol.* 113(2)**,** 313-319. doi: 10.1104/pp.113.2.313.

Guiboileau, A., Sormani, R., Meyer, C., and Masclaux-Daubresse, C. (2010). Senescence and death of plant organs: nutrient recycling and developmental regulation. *C R Biol.* 333(4)**,** 382-391. doi: 10.1016/j.crvi.2010.01.016.

Guo, Y., and Gan, S. (2005). Leaf senescence: signals, execution, and regulation. *Curr Top Dev Biol.* 71**,** 83-112. doi: 10.1016/S0070-2153(05)71003-6.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. *Nature.* 413(6854)**,** 383-389. doi: 10.1038/35096500.

Kim, H.J., Park, J.H., Kim, J., Kim, J.J., Hong, S., Kim, J., et al. (2018a). Time-evolving genetic networks reveal a NAC troika that negatively regulates leaf senescence in Arabidopsis. *Proc Natl Acad Sci U. S. A.* 115(21)**,** 4930-4939. doi: 10.1073/pnas.1721523115.

Kim, J., Kim, J.H., Lyu, J.I., Woo, H.R., and Lim, P.O. (2018b). New insights into the regulation of leaf senescence in Arabidopsis. *J Exp Bot.* 69(4)**,** 787-799. doi: 10.1093/jxb/erx287.

Kim, J., Kim, Y., Yeom, M., Kim, J.H., and Nam, H.G. (2008). FIONA1 is essential for regulating period length in the Arabidopsis circadian clock. *Plant Cell.* 20(2)**,** 307-319. doi: 10.1105/tpc.107.055715.

Kim, J., Park, S.J., Lee, I.H., Chu, H., Penfold, C.A., Kim, J.H., et al. (2018c). Comparative transcriptome analysis in Arabidopsis ein2/ore3 and ahk3/ore12 mutants during dark-induced leaf senescence. *J Exp Bot.* 69(12)**,** 3023-3036. doi: 10.1093/jxb/ery137.

Kim, J., and Somers, D.E. (2010). Rapid assessment of gene function in the circadian clock using artificial microRNA in Arabidopsis mesophyll protoplasts. *Plant Physiol.* 154(2)**,** 611-621. doi: 10.1104/pp.110.162271.

Kim, J.H., Woo, H.R., Kim, J., Lim, P.O., Lee, I.C., Choi, S.H., et al. (2009). Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. *Science* 323(5917)**,** 1053-1057. doi: 10.1126/science.1166386.

Koo, J.C., Lee, I.C., Dai, C., Lee, Y., Cho, H.K., Kim, Y., et al. (2017). The protein trio RPK1-CaM4-RbohF mediates transient superoxide production to trigger age-dependent cell death in Arabidopsis. *Cell Rep.* 21(12)**,** 3373-3380. doi: 10.1016/j.celrep.2017.11.077.

Lee, I.C., Hong, S.W., Whang, S.S., Lim, P.O., Nam, H.G., and Koo, J.C. (2011). Age-dependent action of an ABA-inducible receptor kinase, RPK1, as a positive regulator of

senescence in Arabidopsis leaves. *Plant Cell Physiol.* 52(4)**,** 651-662. doi: 10.1093/pcp/pcr026.

Lehmann, S., Dominguez-Ferreras, A., Huang, W.J., Denby, K., Ntoukakis, V., and Schafer, P. (2020). Novel markers for high-throughput protoplast-based analyses of phytohormone signaling. *PLoS One.* 15(6)**,** e0234154. doi: 10.1371/journal.pone.0234154.

Li, N., Uhrig, J.F., Thurow, C., Huang, L.J., and Gatz, C. (2019). Reconstitution of the jasmonate signaling pathway in plant protoplasts. *Cells.* 8(12). doi: 10.3390/cells8121532.

Lim, P.O., Kim, Y., Breeze, E., Koo, J.C., Woo, H.R., Ryu, J.S., et al. (2007). Overexpression of a chromatin architecture-controlling AT-hook protein extends leaf longevity and increases the post-harvest storage life of plants. *Plant J.* 52(6)**,** 1140-1153. doi: 10.1111/j.1365-313X.2007.03317.x.

Lim, P.O., Woo, H.R., and Nam, H.G. (2003). Molecular genetics of leaf senescence in Arabidopsis. *Trends Plant Sci.* 8(6)**,** 272-278. doi: 10.1016/S1360-1385(03)00103-1.

Munne-Bosch, S., and Alegre, L. (2002). Plant aging increases oxidative stress in chloroplasts. *Planta.* 214(4)**,** 608-615. doi: 10.1007/s004250100646.

Noh, Y.S., and Amasino, R.M. (1999). Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Mol Biol.* 41(2)**,** 181-194. doi: 10.1023/a:1006342412688.

Ossowski, S., Schwab, R., and Weigel, D. (2008). Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J.* 53(4)**,** 674-690. doi: 10.1111/j.1365-313X.2007.03328.x.

Rauf, M., Arif, M., Dortay, H., Matallana-Ramirez, L.P., Waters, M.T., Gil Nam, H., et al. (2013). ORE1 balances leaf senescence against maintenance by antagonizing G2-like-mediated transcription. *EMBO Rep.* 14(4)**,** 382-388. doi: 10.1038/embor.2013.24.

Rolland, V. (2018). Determining the subcellular localization of fluorescently tagged proteins using protoplasts extracted from transiently transformed Nicotiana benthamiana leaves. *Methods Mol Biol.* 1770**,** 263-283. doi: 10.1007/978-1-4939-7786-4_16.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell.* 18(5)**,** 1121-1133. doi: 10.1105/tpc.105.039834.

Thomas, H., Ougham, H.J., Wagstaff, C., and Stead, A.D. (2003). Defining senescence and death. *J Exp Bot.* 54(385)**,** 1127-1132. doi: 10.1093/jxb/erg133.

Tyurin, A.A., Suhorukova, A.V., Kabardaeva, K.V., and Goldenkova-Pavlova, I.V. (2020). Transient gene expression is an effective experimental tool for the research into the fine mechanisms of plant gene function: Advantages, limitations, and solutions. *Plants.* 9(9)**,** 1187.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., et al. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Res.* 40(15)**,** e115. doi: 10.1093/nar/gks596.

Vachova, H., Alquicer, G., Sedinova, M., Sachova, J., Hradilova, M., and Varga, V. (2020). A rapid approach for in locus overexpression of Trypanosoma brucei proteins. *Mol Biochem Parasitol.* 239**,** 111300. doi: 10.1016/j.molbiopara.2020.111300.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* 33(5)**,** 949-956. doi: 10.1046/j.1365-313x.2003.01676.x.

Wang, P., Richter, A.S., Kleeberg, J.R.W., Geimer, S., and Grimm, B. (2020). Post-translational coordination of chlorophyll biosynthesis and breakdown by BCMs maintains chlorophyll homeostasis during leaf development. *Nat Commun.* 11(1)**,** 1254. doi: 10.1038/s41467-020-14992-9.

Wang, P., Yin, L., Liang, D., Li, C., Ma, F., and Yue, Z. (2012). Delayed senescence of apple leaves by exogenous melatonin treatment: toward regulating the ascorbate-glutathione cycle. *J Pineal Res.* 53(1)**,** 11-20. doi: 10.1111/j.1600-079X.2011.00966.x.

Woo, H.R., Chung, K.M., Park, J.H., Oh, S.A., Ahn, T., Hong, S.H., et al. (2001). ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. *Plant Cell.* 13(8)**,** 1779-1790. doi: 10.1105/TPC.010061.

Woo, H.R., Goh, C.H., Park, J.H., Teyssendier de la Serve, B., Kim, J.H., Park, Y.I., et al. (2002). Extended leaf longevity in the ore4-1 mutant of Arabidopsis with a reduced expression of a plastid ribosomal protein gene. *Plant J.* 31(3)**,** 331-340. doi: 10.1046/j.1365-313x.2002.01355.x.

Woo, H.R., Kim, H.J., Lim, P.O., and Nam, H.G. (2019). Leaf senescence: systems and dynamics aspects. *Annu Rev Plant Biol.* 70**,** 347-376. doi: 10.1146/annurev-arplant-050718-095859.

Woo, H.R., Kim, J.H., Kim, J., Kim, J., Lee, U., Song, I.J., et al. (2010). The RAV1 transcription factor positively regulates leaf senescence in Arabidopsis. *J Exp Bot.* 61(14)**,** 3947-3957. doi: 10.1093/jxb/erq206.

Woo, H.R., Koo, H.J., Kim, J., Jeong, H., Yang, J.O., Lee, I.H., et al. (2016). Programming of plant leaf senescence with temporal and inter-organellar coordination of transcriptome in Arabidopsis. *Plant Physiol.* 171(1)**,** 452-467. doi: 10.1104/pp.15.01929.

Yoo, S.D., Cho, Y.H., and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc.* 2(7)**,** 1565-1572. doi: 10.1038/nprot.2007.199.

Yu, G., Wang, X., Chen, Q., Cui, N., Yu, Y., and Fan, H. (2019). Cucumber mildew resistance locus O interacts with calmodulin and regulates plant cell death associated with plant immunity. *Int J Mol Sci.* 20(12). doi: 10.3390/ijms20122995.

Zhang, D., Zhang, N., Shen, W., and Li, J.F. (2019). Engineered artificial microRNA precursors facilitate cloning and gene silencing in Arabidopsis and rice. *Int J Mol Sci.* 20(22). doi: 10.3390/ijms20225620.

ACKNOWLEDMENT

Two years run over, I would like to express my sincere thank you to my supervisor, Professor Kim Jeong-sik, who gives me the opportunity to study at Jeju National University, gives me guidance on research, encouragement on study, and inspires the scientific passion to me.

A warm thank you is sent to Doctor Kim Jin-hee for advising my experiments and amending my master manuscript. With her helping, the process of experiments had been sped up and the quality of the thesis had been improved.

After finishing the master course, I also would like to send my warmest thanks to my lab mates in Plant Molecular Genomics laboratory for their supports in my daily life.

Last but not least, I give my appreciation to my family-my dear mother and father, my sister and brother, who are the motivation for me to overcome difficulties and always support and encourage me in my life, especially my sister, who always love and make me become stronger. I also thank Vietnamese friends who share everything during my course and sympathy and help each other whether we are happy or sad.