Plant Diversity 43 (2021) 331–340

Contents lists available at ScienceDirect

Plant Diversity

journal homepage: http://www.keaipublishing.com/en/journals/plant-diversity/
http://journal.kib.ac.cn

Research paper

AtWRKY75 positively regulates age-triggered leaf senescence through gibberellin pathway

Haiyan Zhang [a, d, 1], Liping Zhang [c, 1], Songguo Wu [a, d], Yanli Chen [a, d], Diqiu Yu [a, e, **], Ligang Chen [a, b, *]

[a] CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan, 650223, China
[b] Center of Economic Botany, Core Botanical Gardens, Chinese Academy of Sciences, Menglun, Mengla, Yunnan, 666303, China
[c] CAS Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, No. 132, Lanhei Road, Kunming, Yunnan, 650201, China
[d] University of Chinese Academy of Sciences, Beijing, 100049, China
[e] State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, Yunnan, 650091, China

ARTICLE INFO

Article history:
Received 12 June 2020
Received in revised form
18 October 2020
Accepted 19 October 2020
Available online 1 November 2020

Keywords:
WRKY75
Leaf senescence
GA
DELLAs

ABSTRACT

WRKY transcription factors play essential roles during leaf senescence. However, the mechanisms by which they regulate this process remains largely unknown. Here, we identified the transcription factor WRKY75 as a positive regulator during leaf senescence. Mutations of *WRKY75* caused a delay in age-triggered leaf senescence, whereas overexpression of *WRKY75* markedly accelerated this process. Expression of senescence-associated genes (*SAGs*) was suppressed in *WRKY75* mutants but increased in *WRKY75*-overexpressing plants. Further analysis demonstrated that WRKY75 directly associates with the promoters of *SAG12* and *SAG29*, to activate their expression. Conversely, GAI and RGL1, two DELLA proteins, can suppress the WRKY75-mediated activation, thereby attenuating *SAG* expression during leaf senescence. Genetic analyses showed that *GAI* gain-of-function or *RGL1* overexpression can partially rescue the accelerated senescence phenotype caused by *WRKY75* overexpression. Furthermore, WRKY75 can positively regulate *WRKY45* expression during leaf senescence. Our data thus imply that *WRKY75* may positively modulate age-triggered leaf senescence through the gibberellin-mediated signaling pathway.

1. Introduction

Plants undergo developmental and physiological changes throughout their life history, ending with senescence and death (Lim et al., 2005). Leaf senescence is an important part of plant development and increases reproductive success. During senescence, plants relocate mobilizable nutrients from older leaves to other developing organs, including seeds, stems and roots (Lim et al., 2005). Under optimal conditions, the onset of leaf senescence is normally initiated in an age-dependent manner; however, it can also be triggered by environmental changes that are integrated into the developmental aging program (Buchanan-Wollaston et al., 2005; Lim et al., 2007). Plant senescence represents one of the adaptive mechanisms that plants possess that may help to increase survival in a given ecological niche.

Leaf senescence is a highly coordinated and sophisticated cellular process during which leaf cells undergo active degenerative alterations. Furthermore, this biochemical process is closely associated with the increased expression of numerous senescence-associated genes (*SAGs*), including *SAG12* and *SAG29*, but reduced expression of photosynthetic genes, such as *CAB1* and *RBCS1A* (Gan and Amasino, 1995; Hortensteiner, 2006; Park et al., 1998; Qi et al., 2015; Weaver et al., 1998). Studies have also

* Corresponding author. CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan, 650223, China. Fax: +86 871 65160916.
** Corresponding author. CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan, 650223, China. Fax: +86 871 65160916.
E-mail addresses: ydq@ynu.edu.cn (D. Yu), chenligang@xtbg.ac.cn (L. Chen).
Peer review under responsibility of Editorial Office of Plant Diversity.
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.pld.2020.10.002

demonstrated that a distinct set of phytohormones play critical roles during leaf senescence, with jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), salicylic acid (SA), brassinosteroids accelerating senescence, while auxin and cytokinins delay leaf aging (Jibran et al., 2013). Interestingly, gibberellin (GA) was recently revealed to be a crucial growth regulator that positively modulates leaf senescence in *Arabidopsis* (Chen et al., 2014, 2017).

Temporal profiling has revealed that leaf senescence involves the coordinated expression of thousands of genes. Notably, numerous *WRKY* genes are strongly expressed in senescing leaves, suggesting that *WRKY* genes play a role in leaf senescence (Guo et al., 2004). Recent studies have provided evidence that WRKY proteins function as critical components in senescence-associated regulatory pathways. For example, WRKY57 was shown to function as a common component of JA- and auxin-mediated signaling pathways to modulate JA-induced leaf senescence (Jiang et al., 2014). In addition, WRKY75, together with SA and reactive oxygen species (ROS), has been shown to form a tripartite amplification loop to promote leaf senescence (Guo et al., 2017). Furthermore, our recent study demonstrated that WRKY45 directly binds several *SAG* promoters to activate their expression, thereby activating GA-mediated leaf senescence (Chen et al., 2017).

Here, we show that *WRKY75* transcript and protein levels increased during leaf senescence. We found that age-triggered leaf senescence was delayed in *WRKY75* mutants, but accelerated in plants overexpressing *WRKY75*. Further investigation indicated that WRKY75 participates in GA-mediated leaf senescence by directly regulating the expression of several downstream *SAGs*. We also show that the ability of WRKY75 to activate transcription can be repressed by both GAI and RGL1, and that *GAI* gain-of-function or *RGL1* overexpression can partially rescue the early-senescence phenotype caused by *WRKY75* overexpression. Our findings thus demonstrate that WRKY75 may participate in GA-mediated leaf senescence in *Arabidopsis*.

2. Materials and methods

2.1. Materials and plant growth conditions

All mutant and transgenic plants were in the Col-0 background. The *WRKY* mutants (*wrky75-1* and *wrky75-25*), *WRKY75* and *RGL1* transgenic over-expressing plants, and *Myc-WRKY75/RGL1* plants have been described in our previous studies (Chen et al., 2017; Zhang et al., 2018). *Arabidopsis* plants were grown at 22 °C under a 16-h-light/8-h-dark cycle. All chemicals were purchased from Takara Biotechnology (Dalian, China).

2.2. qRT-PCR analysis

Quantitative RT-PCR was conducted as described in Chen et al. (2013). The gene-specific primers for qRT-PCR were *WRKY75-F* (5′-ATATGGCCAAAAGGCCGTCA-3′) and *WRKY75-R* (5′-TGCTCGAAGTTTTCGGTGGA-3′), *SAG12-F* (5′-ATCCAAAAGCAACTTCTATTACAGG-3′) and *SAG12-R* (5′-CCACTGCCTTCATCAGTGC-3′), *SAG29-F* (5′-GCCACCAGGGAGAAAAGG-3′) and.

SAG29-R (5′-CCACGAAATGTGTTACCATTAGAA-3′), *ACTIN2-F* (5′-TGTGCCAATCTACGAGGGTTT-3′) and *ACTIN2-R* (5′-TTTCCCGCTCTGCTGTTGT-3′).

2.3. Measurements of chlorophyll content and ion leakage

Chlorophyll was extracted from detached leaves with 80% acetone. Chlorophyll content was determined at 663 and 645 nm according to Lichtenthaler (1987).

To measure ion leakage, we incubated detached leaves in deionized water for at least 2 h (less than 10 h) and then determined conductivities (C1) of the solutions. The samples were then boiled in the same deionized water for 15 min. After cooling, the conductivities (C2) of the solutions were measured again (Li et al., 2013). The degree of ion leakage was calculated as the ratio of C1:C2.

2.4. ChIP assays

WRKY75:YFP-WRKY75:3′-WRKY75, *Myc-WRKY75/RGL1* and Col-0 leaves were harvested for ChIP experiments as described in Saleh et al. (2008). The GFP or Myc antibody was used to immunoprecipitate the protein–DNA complex, and the precipitated DNA was purified using a PCR purification kit for real-time qPCR analysis. The ChIP experiments were performed three times. Chromatin precipitated without antibody was used as the negative control, while isolated chromatin before precipitation was used as the input control. ChIP results are presented as a percentage of input DNA.

2.5. Transient expression assays

Transient expression assays were performed in *Nicotiana benthamiana* leaves as described by Chen et al. (2017). Briefly, NLS was fused with a GFP reporter gene behind the native promoter of *SAG12* or *SAG29*.The *CaMV* 35S promoter was used to drive full-length coding sequences of *RGL1*, *GAI*, *GUS*, and *WRKY75*. These constructs were then introduced into *Agrobacterium tumefaciens* (strain EHA105). Expression of *GFP* was determined 48 h after infiltration. Experiments were repeated three times with similar results.

2.6. Determination of YFP

Senescing leaves of *WRKY75:YFP-WRKY75:3′-WRKY75* plants were detached and then YFP was observed under a confocal laser scanning microscope (Olympus).

2.7. Treatment of GA

We treated seedlings with GA_3 as described in Chen et al. (2017). *Myc-WRKY75/RGL1* seedlings were harvested after treatment with 50 μM GA_3 or mock for 8 h.

2.8. Accession numbers

The following genes were detected in this work: *WRKY75* (At5G13080), *WRKY45* (At3G01970), *RGL1* (At1G66350), *GAI* (At1G14920), *SAG12* (At5G45890), *SAG29* (At5g13070), and *ACTIN2* (At3G18780).

3. Results

3.1. Expression of WRKY75 in senescing leaves

WRKY75 was previously found to play important roles in phosphate starvation, defense responses, flowering initiation, and root hair initiation (Devaiah et al., 2007; Encinas-Villarejo et al., 2009; Zhang et al., 2018; Rishmawi et al., 2014). An *Arabidopsis* microarray database indicates that *WRKY75* is highly expressed in senescent leaves (Winter et al., 2007; Fig. S1). Our data further confirm these results (Fig. 1), and imply that *WRKY75* may function as a *SAG* that is up-regulated during senescence. *WRKY75* expression increased greatly in leaves during early senescence (ES), and showed even stronger expression at late senescence (LS) (Fig. 1A

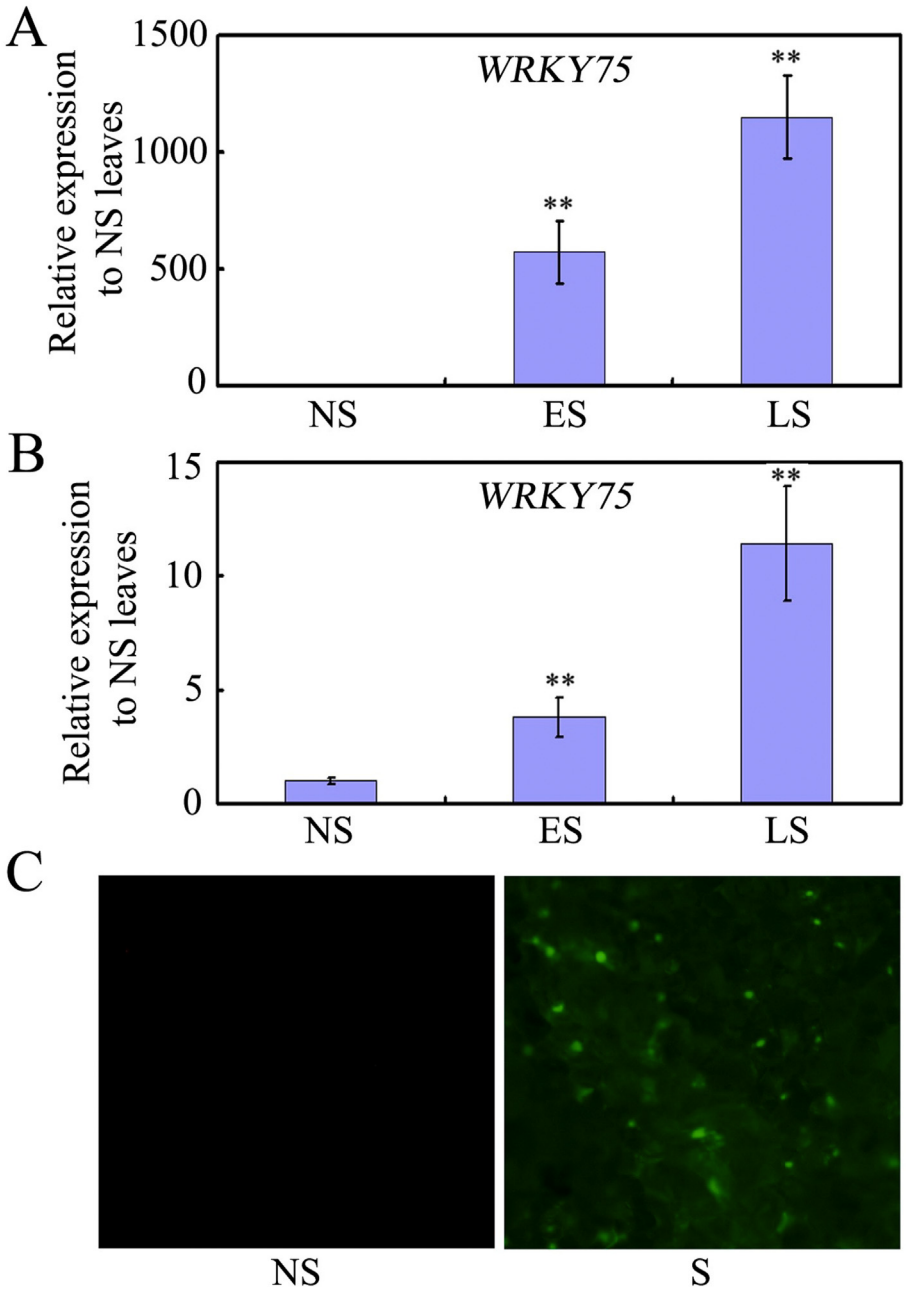

Fig. 1. Expression of *WRKY75* in senescing leaves. (A) qRT-PCR analysis of *WRKY75* transcript levels in wild-type leaves at different developmental stages. Transcript levels of WRKY75 in non-senescent (NS) leaves were arbitrarily set to 1. Values are means ± SD of three independent biological replicates. $^{**}P < 0.01$, Student's t-test compared with NS leaves. (B) qRT-PCR analysis of *WRKY75* transcript levels in different parts of a senescing wild-type leaf. Transcript levels of WRKY75 in NS leaves were arbitrarily set to 1. Values are means ± SD of three independent biological replicates. $^{**}P < 0.01$, Student's t-test compared with NS parts of a senescing wild-type leaf. (C) YFP detection of WRKY75 in *wrky75-25* mutant background that harbors the *WRKY75:YFP-WRKY75:3′-WRKY75*. YFP signals were observed in senescing leaves of the *WRKY75:YFP-WRKY75:3′-WRKY75* transgenic plants. These experiments were performed three times with similar results.

and Fig. S2A). This same pattern of *WRKY75* expression was observed across the senescence gradient from the tip to the base of leaves (Fig. 1B and Fig. S2B).

To further understand *WRKY75* expression patterns, we measured WRKY75 protein accumulation during leaf senescence by visualizing yellow fluorescent protein (YFP) in leaves of *WRKY75-$_{pro}$:YFP-WRKY75:3′WRKY75* plants. No YFP signal was observed in non-senescing leaves, whereas strong YFP signals were observed in senescing leaves (Fig. 1C). These findings show that both transcript and protein levels of WRKY75 increase during leaf senescence, suggesting that WRKY75 plays a role in leaf senescence.

3.2. Altered age-triggered leaf senescence resulting from knock-down or ectopic expression of WRKY75

High expression levels of *WRKY75* in senescing leaves prompted us to examine the role of *WRKY75* in senescence using *WRKY75* mutants (*wrky75-1* and *wrky75-25*) (Zhang et al., 2018). Analysis of the rosettes of 7-week-old plants revealed that leaf senescence was delayed in *WRKY75* mutants compared to in WT plants (Fig. 2A).This finding is consistent with that of two recent reports (Guo et al., 2017; Zhang et al., 2017). Chlorophyll content in these plants indicated that chlorophyll was degraded more slowly in

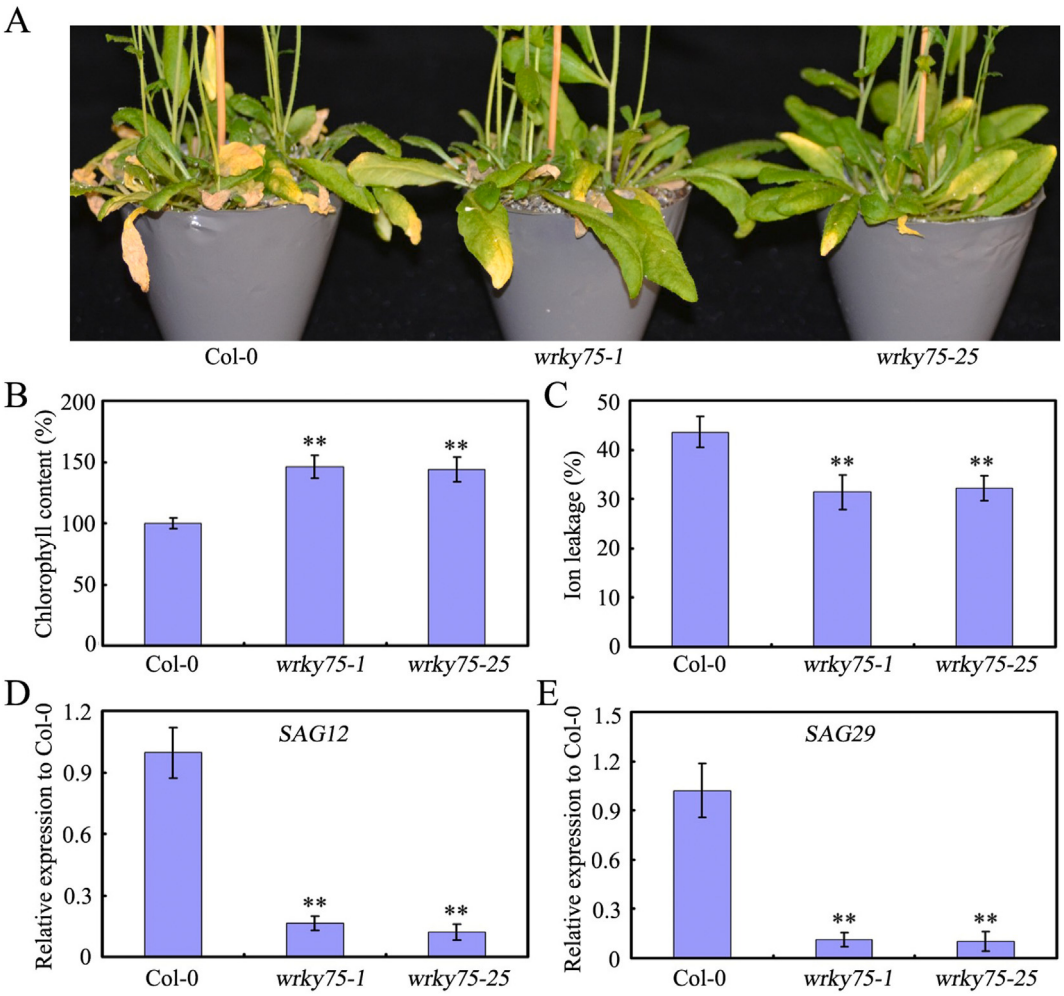

Fig. 2. *WRKY75* mutation delays age-triggered leaf senescence. (A) The senescence phenotypes of 7-week-old Col-0 and *wrky75* mutant plants. (B) Relative chlorophyll content of the Col-0 and *wrky75* mutant plants. (C) Membrane ion leakage of the Col-0 and *wrky75* mutant plants. (D) and (E) qRT-PCR analysis of transcript levels of senescence marker genes in age-triggered senescing leaves of Col-0 and *wrky75* mutant plants. For B-E, values are means ± SD of three independent biological replicates. $^{**}P < 0.01$, Student's t-test compared with Col-0. These experiments were performed three times with similar results.

WRKY75 mutants than in WT plants (Fig. 2B). Correspondingly, both ion leakage and expression of representative senescence-induced *SAGs* (e.g., *SAG12* and *SAG29*) were lower in *WRKY75* mutants than in WT plants (Fig. 2C–E). These findings indicate that disruption of *WRKY75* delays the senescence process of plants.

To further determine the role of *WRKY75* in leaf senescence, we again used *35S:WRKY75* transgenic *Arabidopsis* plants (Zhang et al., 2018). Compared with WT plants, *35S:WRKY75* transgenic plants showed early onset senescence (Fig. S3A). Consistent with this finding, transgenic plants had lower chlorophyll contents, but higher ion leakage and stronger *SAG* expression (Fig. S3B–E). Together, these observations suggest that over-expression of *WRKY75* promotes leaf senescence.

3.3. *In vivo interactions between WRKY75 and its target promoters*

Previous studies have revealed that WRKY proteins function by directly binding to a *cis*-acting DNA element, namely W-box (T/CTGACC/T), present in promoters of their target genes (Eulgem et al., 2000; Ulker and Somssich, 2004). We provide evidence that *WRKY75* functions as an activator in age-triggered leaf senescence by promoting the expression of senescence-associated genes. We found several putative W-box elements in promoters of both *SAG12* and *SAG29* (Fig. 3A), indicating that WRKY75 may directly regulate their expression, thus promoting leaf senescence. To determine whether WRKY75 can directly regulate *SAG12* or *SAG29* expression, chromatin immunoprecipitation (ChIP) experiments were performed using *WRKY75:YFP-WRKY75:3′-WRKY75* plants (Rishmawi et al., 2014). These experiments showed that WRKY75 can interact with the *SAG12* and *SAG29* promoters (*pSAG12-3*, *pSAG29-2*, and *pSAG29-3*) via the W-box sequence (Fig. 3B), suggesting that WRKY75 directly regulates their transcription.

To further determine the regulatory function of *WRKY75*, we performed transient expression assays in tobacco (*N. benthamiana*) leaves. We fused the promoters of *SAG12* and *SAG29*—both direct targets of WRKY75—to a reporter construct, the nuclear localization signal (NLS)-GFP gene (*SAG12:NLS-GFP* and *SAG29:NLS-GFP*) (Fig. 3C). The effector plasmids had a *GUS* or *WRKY75* gene under the control of the *CaMV* 35S promoter (*35S:GUS* and *35S:WRKY75*) (Fig. 3C). Compared to controls, co-expression of *WRKY75* in the effector plasmids greatly increased *GFP* reporter expression

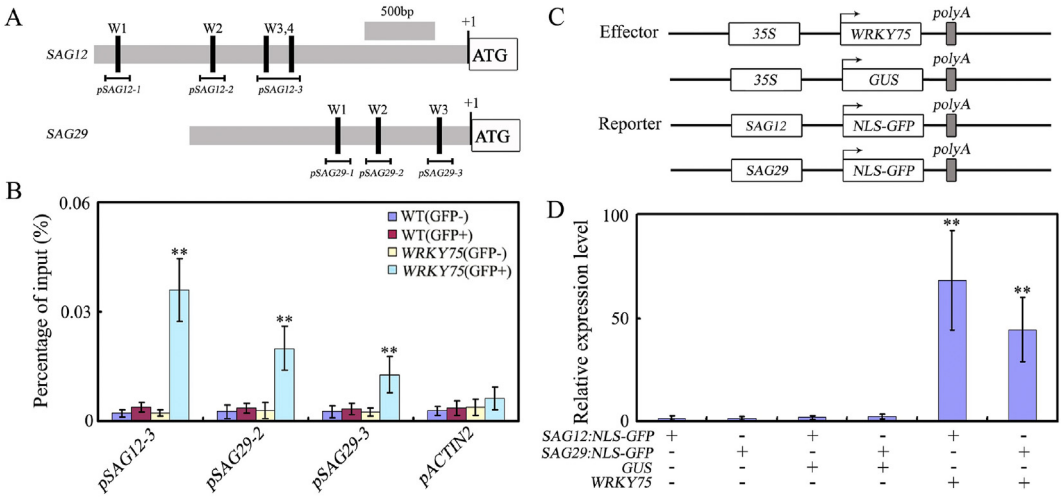

Fig. 3. WRKY75 directly regulates the expression of *SAG12* and *SAG29*. (A) The promoter structure of the *SAG12* and *SAG29* genes and fragment used in the ChIP assay. (B) WRKY75 directly binds to the promoters of *SAG12* and *SAG29*. ChIP assays were performed with chromatin prepared from *WRKY75:YFP-WRKY75:3′-WRKY75* transgenic plants, using an anti-GFP antibody (IP). ChIP results are presented as a percentage of input DNA. Values are mean ± SD of three independent biological replicates. Asterisks indicate Student's t-test significant differences as compared to controls, $^{**}P < 0.01$. (C) Schematic of the *SAG12:NLS-GFP* and *SAG29:NLS-GFP* reporters and *WRKY75* and *GUS* effectors. (D) Transient expression assays showed that WRKY75 activates the expression of *SAG12* and *SAG29* determined by qRT-PCR analysis. Values are means ± SD of three independent biological replicates. $^{**}P < 0.01$, Student's t-test compared with controls. These experiments were performed three times with similar results.

(Fig. 3D), suggesting that WRKY75 functions as an activator during age-triggered leaf senescence.

3.4. RGL1 and GAI inhibit WRKY75-mediated transcriptional activation

We previously reported that WRKY75 can physically interact with DELLA proteins (Zhang et al., 2018), prompting us to hypothesize that WRKY75 may participate in senescence through the GA pathway. We also speculated that physical interactions between WRKY75 and DELLA proteins inhibit the ability of WRKY75 to activate transcription of target genes. To test these hypotheses, we again performed transient expression assays in tobacco (*N. benthamiana*) leaves with *35S:WRKY75*, *35S:RGL1*, *35S:GAI* and *35S:GUS* as effectors and *SAG12:NLS-GFP* as a reporter (Fig. 4A). We found that *WRKY75* expression greatly increases the expression of *GFP* driven by the *SAG12* promoter. However, co-expression of *RGL1* or *GAI* with *WRKY75* markedly reduces *GFP* expression in comparison with expression of *WRKY75* or *GUS* alone (Fig. 4B). These results demonstrate that both GAI and RGL1 can repress WRKY75-mediated transcriptional activation.

To determine whether the ability of WRKY75 to bind to target genes is affected by interactions with DELLA proteins, we performed ChIP assays using *Myc-WRKY75/RGL1* plants. When we treated plants with GA, the binding efficiency of WRKY75 to the *SAG12* promoter increased greatly, implying that RGL1 may reduce the binding ability of WRKY75 to its target genes *in vivo*.

3.5. Repression of age-triggered leaf senescence by GAI gain-of-function or RGL1 overexpression

Because several DELLAs physically associate with WRKY75 and modulate its transcriptional ability, we then wondered whether *GAI* gain-of-function or *RGL1* overexpression could rescue the earlier senescence phenotype caused by *WRKY75* overexpression. Then both *Myc-WRKY75/gai-1* and *Myc-WRKY75/RGL1* plants were used (Zhang et al., 2018). Senescence occurred earlier in *Myc-WRKY75/gai-1* plants than in *gai-1* plants (Fig. 5A). Furthermore, *Myc-WRKY75/gai-1* plants had lower chlorophyll content and higher levels of *SAG* expression than *gai-1* plants (Fig. 5B–E). *Myc-WRKY75/RGL1* plants showed similar phenotypes (Fig. S4). These findings indicate that *GAI* gain-of-function or *RGL1* overexpression may at least partially delay the early senescence phenotype caused by *WRKY75* overexpression.

3.6. Role of WRKY75 in GA-mediated leaf senescence

To further determine the role of WRKY75 in GA-mediated leaf senescence, we compared the GA response in *wrky75-1*, *wrky75-25*, WT, *35S:WRKY75-L3*, and *35S:WRKY75-L6* plants. We previously demonstrated that GA induces slight increases in *WRKY75* gene expression (Zhang et al., 2018). Here, we confirm that *WRKY75* expression is promoted in *della* but reduced in *ga1* or *gid1a/gid1b/gid1c* plants (Fig. 6A). Furthermore, when we treated plants with GA, chlorophyll content and *SAG12* expression indicated that GA-mediated leaf senescence was delayed in *wrky75* mutants but accelerated in *WRKY75* overexpressing plants. Combined, these results suggest that *WRKY75* participates in the modulation of leaf senescence through the GA pathway.

3.7. WRKY75 positively regulates WRKY45 expression during leaf senescence

Our previous study revealed that WRKY45 functions as a novel activator of the senescence transcriptional network (Chen et al., 2017). Thus, we wondered whether WRKY75 would affect the expression of *WRKY45* during leaf senescence. As shown in Fig. 7A, *WRKY45* expression was inhibited in the *wrky75* mutants but was activated in *WRKY75* over-expressing plants (Fig. 7A). To test whether WRKY45 is a direct target of *WRKY75*, ChIP assays were preformed using *WRKY75:YFP-WRKY75:3′-WRKY75* plants. WRKY75 can directly bind to the *WRKY45* promoter through the W-box cis-element (*pWRKT45-2*) (Fig. 7B and C). These results demonstrate that WRKY75 directly regulates *WRKY45* expression during leaf senescence, and also imply that there may exist extensive cross-regulation among WRKY members during leaf senescence, which contributes to the facilitation of senescence-associated transcriptional reprogramming.

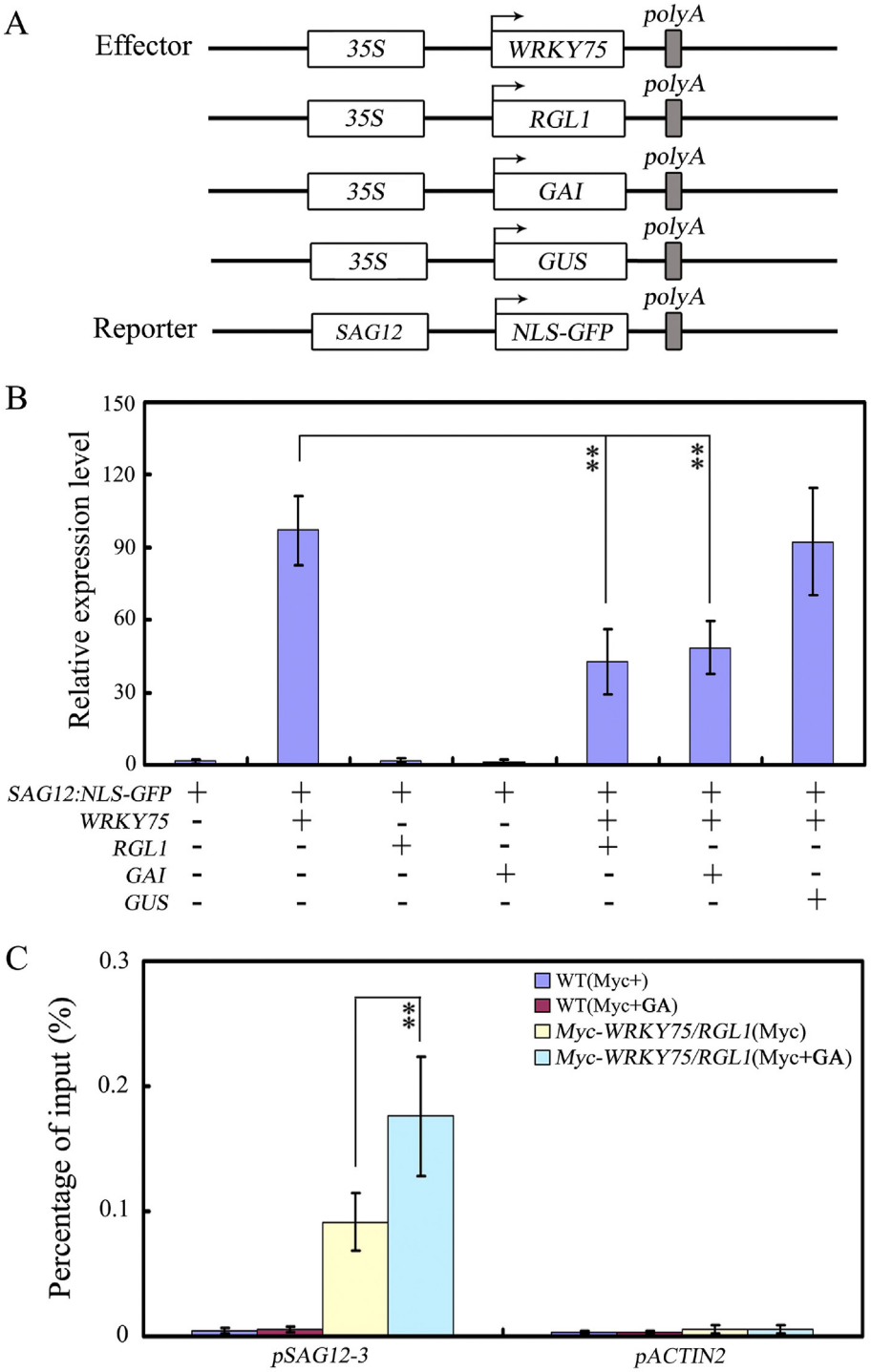

Fig. 4. GAI and RGL1 repress WRKY75 activation ability. (A) Schematic of the *SAG12:NLS-GFP* reporter and *WRKY75*, *RGL1*, *GAI* and *GUS* effectors. (B) Transient expression assays showed that RGL1 and GAI repress transcriptional activation of WRKY75 determined by qRT-PCR analysis. Values are mean ± SD of three independent biological replicates. Asterisks indicate Student's t-test significant differences as compared to controls, $^{**}P < 0.01$. (C) RGL1 interferes the binding of WRKY75 to its target genes (shown in Fig. 4A). ChIP assays were performed with chromatin prepared from *Myc-WRKY75/RGL1* plants, using an anti-*Myc* antibody (IP). ChIP results are presented as a percentage of input DNA. Values are mean ± SD of three independent biological replicates. Asterisks indicate Student's t-test significant differences as compared to controls, $^{**}P < 0.01$. These experiments were performed three times with similar results.

4. Discussion

Plant senescence is tightly controlled by the temporal coordinated expression of numerous *SAGs*. Over the past decades, our understanding of plant senescence has improved immensely. Microarray expression profiling has identified WRKY factors as the second largest transcription factor group in the senescent transcriptome (Guo et al., 2004), suggesting that these genes play central roles in modulating transcriptional changes during senescence. However, the biological function of WRKY factors during leaf senescence remains largely unknown. Recently, several *Arabidopsis* WRKY proteins have been shown to play important roles during

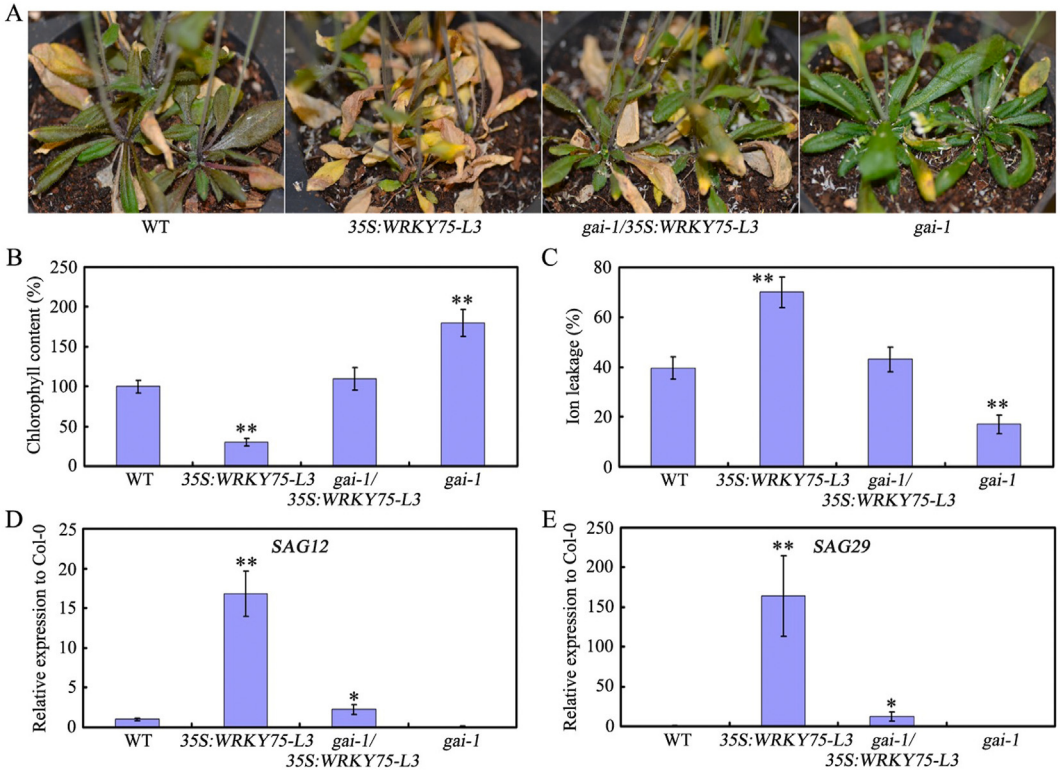

Fig. 5. *GAI* gain-of-function partially rescues the early-senescence phenotype of *WRKY75* overexpressing plants. (A) The senescence phenotypes of the indicated genotypes. (B) Relative chlorophyll content of the indicated genotypes. (C) Membrane ion leakage of the indicated genotypes. (D) and (E) qRT-PCR analysis of transcript levels of senescence marker genes in the indicated genotypes. For B-D, values are means ± SD of three independent biological replicates. *$P < 0.05$, **$P < 0.01$, Student's t-test compared with Col-0. These experiments were performed three times with similar results.

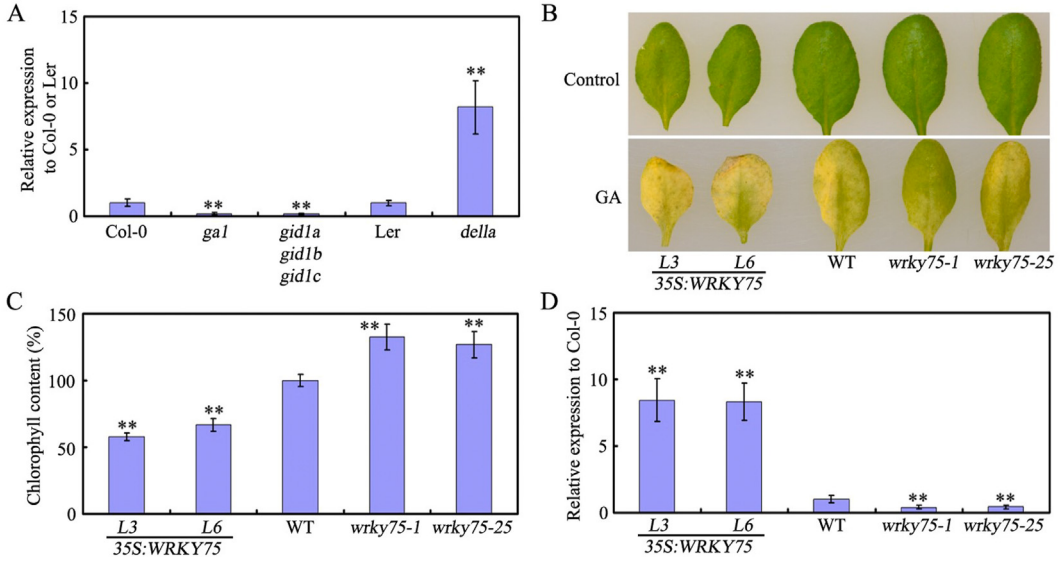

Fig. 6. Effects of GA on *wrky75* and *WRKY75*-overexpressing plants. (A) qRT-PCR analysis of *WRKY75* transcript levels in *ga1*, *gid1agid1bgid1c*, and *della* mutant plants. (B) GA response of the indicated genotypes. (C) Relative chlorophyll content of the indicated genotypes as shown in (B). (D) Relative expression of *SAG12* in the indicated genotypes as treated in (B). For A, C and D, values are means ± SD of three independent biological replicates. **P < 0.01, Student's t-test compared with Col-0 or Ler. These experiments were performed three times with similar results.

leaf senescence, including *WRKY6*, *WRKY22*, *WRKY45*, *WRKY53*, *WRKY54*, *WRKY57*, and *WRKY70* (Robatzek and Somssich 2002; Zhou et al., 2011; Chen et al., 2017; Miao and Zentgraf, 2007; Jiang et al., 2014; Ulker et al., 2007). Here, we provide further evidence that *WRKY75* may function as a new component that positively regulates GA-mediated leaf senescence.

We found that *WRKY75* is strongly expressed in senescing leaves at both mRNA and protein levels, when compared with young

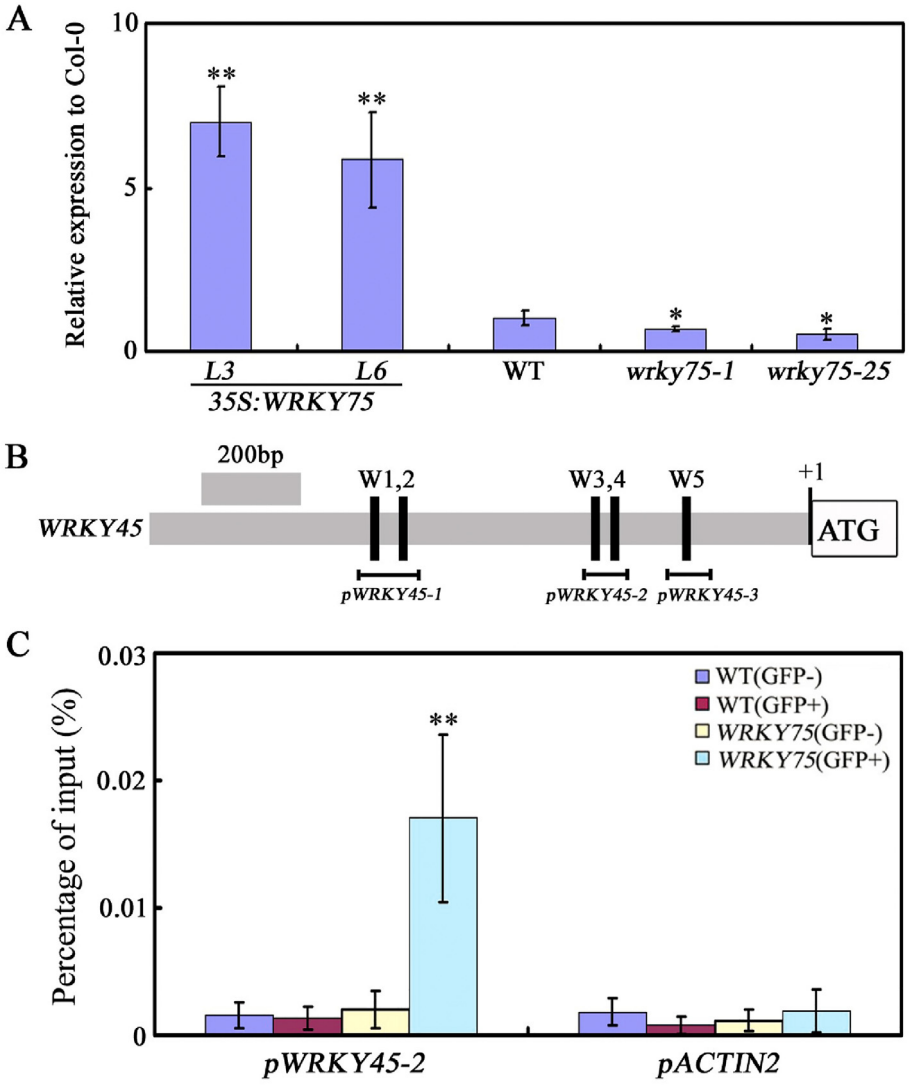

Fig. 7. *WRKY75* positively regulates *WRKY45* expression during leaf senescence. (A) qRT-PCR analysis of transcript levels of *WRKY45* in the indicated genotypes. Values are means ± SD of three independent biological replicates. *P < 0.05, **P < 0.01, Student's t-test compared with Col-0. (B) The promoter structure of *WRKY45* gene and fragment used in the ChIP assay. (C) WRKY75 directly binds to the promoter of *WRKY45*. ChIP assays were performed with chromatin prepared from *WRKY75:YFP-WRKY75:3′-WRKY75* transgenic plants, using an anti-GFP antibody (IP). ChIP results are presented as a percentage of input DNA. Values are mean ± SD of three independent biological replicates. Asterisks indicate Student's t-test significant differences as compared to controls, **P < 0.01. These experiments were performed three times with similar results.

leaves (Fig. 1), implying that *WRKY75* functions as a *SAG* to modulate the senescence. Phenotypic analysis using *wrky75* T-DNA mutants and *WRKY75*-overexpressing plants demonstrated that *WRKY75* acts as a positive regulator during age-triggered leaf senescence (Fig. 2 and Fig. S3). Further investigation revealed that WRKY75 participates in senescence through the direct activation of several *SAG*s, including *SAG12* and *SAG29* (Fig. 3). A recent study also found that WRKY75 directly promotes *SA INDUCTION-DEFICIENT 2* (*SID2*) expression, but suppresses *CATALASE 2* (*CAT2*) transcription during leaf senescence (Guo et al., 2017). Therefore, WRKY75 appears to function as both an activator and repressor to finely modulate leaf senescence. Similarly, our previous study demonstrated that WRKY8 controls plant defense responses to viral infection by positively regulating *ABI4* while negatively regulating *ACS6* (Chen et al., 2013). Taken together, these findings indicate that WRKY factors act as both positive and negative regulators that fine-tune signaling and transcriptional networks involved in mediating plant growth and stress responses.

GA, as an essential hormone, modulates diverse aspects of plant development. Recent studies have demonstrated that GA signaling may have a positive effect on leaf senescence (Chen et al., 2014, 2017·bib_Chen_et_al_2014·bib_Chen_et_al_2017); however, the specific mechanisms by which GA affects this progress have yet to be determined. Numerous studies have suggested that WRKY proteins function as key regulators during leaf senescence. We previously found that WRKY45 regulates age-triggered leaf senescence through a physiological interaction with the DELLA protein RGL1. Other senescence-associated WRKY transcription factors may also be involved in GA-mediated age-dependent leaf senescence. Here, we provide evidence that WRKY75 may positively regulate leaf senescence through the GA pathway.

DELLAs function as crucial transcriptional repressors of the GA pathway, and have been shown to modulate GA-mediated response through interactions with downstream transcription factors. We previously found that GA affects floral initiation via interactions between DELLA and CO or WRKY proteins, including WRKY12,

WRKY13 and WRKY75 (Li et al., 2016; Wang et al., 2016; Zhang et al., 2018). We also found that GA modulates aged-triggered leaf senescence through a physiological interaction with WRKY45 (Chen et al., 2017). The expression of *WRKY75* is markedly elevated at both mRNA and protein levels in senescing leaves, and the physiological interaction between WRKY75 and DELLA proteins may interfere with WRKY75-mediated transcriptional activation (Fig. 4). Furthermore, *RGL1* can disrupt the association of WRKY75 and its target genes *in vivo* (Fig. 4), and *GAI* gain-of-function and *RGL1* overexpression can partially delay the precocious senescence phenotype caused by *WRKY75* overexpression (Figs. 5 and S4). Together, these data suggest that WRKY75 may function downstream of DELLAs to modulate leaf senescence.

In *Arabidopsis*, leaf senescence is tightly associated with flowering, and a delay in flowering always delays senescence. Previous studies have shown that DELLA proteins retard both flowering and senescence in *Arabidopsis*, and that GA signaling positively regulates flowering and senescence by degrading DELLA proteins (Wilson et al., 1992; Achard et al., 2007; Chen et al., 2017). The elimination of DELLA inhibition is thought to accelerate the onset of the reproductive stage, subsequently promoting leaf senescence. Our findings support these results. Specifically, we found that the life cycle of *della* mutants was shortened, whereas the lifespans of the GA biosynthesis mutant *ga1* and the *gid1a/gid1b/gid1c* triple mutants were prolonged. Combined with our previous findings (Zhang et al., 2018), we have demonstrated that flowering and leaf senescence are delayed in *WRKY75* knockout plants and accelerated in plants overexpressing *WRKY75*. We also found that WRKY75 can regulate flowering and leaf senescence through the GA pathway, indicating that WRKY75 may act as a common component of the GA-mediated regulatory network for both flowering and senescence. Identification of regulatory factors like WRKY75 improves our understanding of plant flowering and senescence processes.

5. Conclusions

In this study, we provide evidence that WRKY75 acts as a positive regulator in age-triggered leaf senescence. Our findings suggest that *WRKY75* may modulate the onset and progression of leaf senescence within a senescence-associated transcriptional network by integrating both age and GA signaling. These findings increase our understanding of senescence-associated signaling and transcriptional reprogramming controlled by WRKY proteins.

Author contributions

LGC conceived the project and designed the experiments. HYZ, LPZ, SGW and YLC performed the experiments. LGC wrote the article. All authors interpreted and discussed the data.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

We thank Dong-Tao Ren and Martin Hülskamp for kindly providing the *wrky75-1* and *wrky75-25* mutant seeds, respectively. This work was funded by the National Key R & D Plan (2016YFD0101006), Natural Science Foundation of China (31671275), and Yunnan Fundamental Research Projects (2019FA010).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2020.10.002.

References

Achard, P., Baghour, M., Chapple, A., et al., 2007. The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. Proc. Natl. Acad. Sci. USA 104, 6484–6489.
Buchanan-Wollaston, V., Page, T., Harrison, E., et al., 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. Plant J. 42, 567–585.
Chen, L.G., Xiang, S.Y., Chen, Y.L., et al., 2017. *Arabidopsis* WRKY45 interacts with the DELLA protein RGL1 to positively regulate age-triggered leaf senescence. Mol. Plant 10, 1174–1189.
Chen, L.G., Zhang, L.P., Li, D.B., et al., 2013. WRKY8 transcription factor functions in the TMV-cg defense response by mediating both abscisic acid and ethylene signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 110, E1963–E1971.
Chen, M.,X., Maodzeka, A., Zhou, L.H., et al., 2014. Removal of DELLA repression promotes leaf senescence in *Arabidopsis*. Plant Sci. 219–220, 26–34.
Devaiah, B.N., Karthikeyan, A.S., Raghothama, K.G., 2007. WRKY75 transcription factor is a modulator of phosphate acquisition and root development in *Arabidopsis*. Plant Physiol. 143, 1789–1801.
Encinas-Villarejo, S., Maldonado, A.M., Amil-Ruiz, F., et al., 2009. Evidence for a positive regulatory role of strawberry (*Fragaria* × *ananassa*) Fa WRKY1 and *Arabidopsis* at WRKY75 proteins in resistance. J. Exp. Bot. 60, 3043–3065.
Eulgem, T., Rushton, P.J., Robatzek, S., et al., 2000. The WRKY superfamily of plant transcription factors. Trends Plant Sci. 5, 199–206.
Gan, S., Amasino, R.M., 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270, 1986–1988.
Guo, P.G., Li, Z.H., Huang, P.X., et al., 2017. A tripartite amplification loop involving the transcription factor WRKY75, salicylic acid, and reactive oxygen species accelerates leaf senescence. Plant Cell 29, 2854–2870.
Guo, Y., Cai, Z., Gan, S., 2004. Transcriptome of *Arabidopsis* leaf senescence. Plant Cell Environ. 27, 521–549.
Hortensteiner, S., 2006. Chlorophyll degradation during senescence. Annu. Rev. Plant Biol. 57, 55–77.
Jibran, R., A Hunter, D., P Dijkwel, P., 2013. Hormonal regulation of leaf senescence through integration of developmental and stress signals. Plant Mol. Biol. 82, 547–561.
Jiang, Y.J., Liang, G., Yang, S.Z., et al., 2014. *Arabidopsis* WRKY57 functions as a node of convergence for jasmonic acid- and auxin-mediated signaling in jasmonic acid-induced leaf senescence. Plant Cell 26, 230–245.
Li, Z., Peng, J., Wen, X., et al., 2013. Ethylene-insensitive3 is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in *Arabidopsis*. Plant Cell 25, 3311–3328.
Lim, P.O., Kim, H.J., Nam, H.G., 2005. Leaf senescence. Annu. Rev. Plant Biol. 58, 115–136.
Li, W., Wang, H.P., Yu, D.Q., 2016. The *Arabidopsis* WRKY transcription factors WRKY12 and WRKY13 oppositely regulate flowering under short-day conditions. Mol. Plant 9, 1492–1503.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.
Miao, Y., Zentgraf, U., 2007. The antagonist function of *Arabidopsis* WRKY53 and ESR/ESP in leaf senescence is modulated by the jasmonic and salicylic acid equilibrium. Plant Cell 19, 819–830.
Park, J.H., Oh, S.A., Kim, Y.H., et al., 1998. Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in *Arabidopsis*. Plant Mol. Biol. 37, 445–454.
Qi, T.C., Wang, J.J., Huang, H., et al., 2015. Regulation of jasmonate-induced leaf senescence by antagonism between bHLH subgroup IIIe and IIId factors in *Arabidopsis*. Plant Cell 27, 1634–1649.
Rishmawi, L., Pesch, M., Juengst, C., et al., 2014. Non-cell-autonomous regulation of root hair patterning genes by WRKY75 in Arabidopsis. Plant Physiol. 165, 186–195.
Robatzek, S., Somssich, I.E., 2002. Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev. 16, 1139–1149.
Saleh, A., Alvarez-Venegas, R., Avramova, A., 2008. An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants. Nat. Protoc. 3, 1018–1025.
Ulker, B., Shahid Mukhtar, M., Somssich, I.E., 2007. The WRKY70 transcription factor of *Arabidopsis* influences both the plant senescence and defense signaling pathways. Planta 226, 125–137.
Ulker, B., Somssich, I.E., 2004. WRKY transcription factors: from DNA binding towards biological function. Curr. Opin. Plant Biol. 7, 491–498.
Wang, H., Pan, J.J., Li, Y., et al., 2016. The DELLA-CONSTANS transcription factor cascade integrates gibberelic acid and photoperiod signaling to regulate flowering. Plant Physiol. 172, 479–488.

Weaver, L.M., Gan, S., Quirino, B., et al., 1998. A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol. Biol. 37, 455–469.
Wilson, R.N., Heckman, J.W., Somerville, C.R., 1992. Gibberellin is required for floweringin *Arabidopsis thaliana* under short days. Plant Physiol. 100, 403–408.
Winter, D., Vinegar, B., Nahal, H., et al., 2007. An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One 2, e718.
Zhang, L.P., Chen, L.G., Yu, D.Q., 2018. Transcription factor WRKY75 interacts with DELLA proteins to affect flowering. Plant Physiol. 176, 790–803.
Zhang, S.C., Li, C., Wang, R., et al., 2017. The *Arabidopsis* mitochondrial protease FtSH4 is involved in leaf senescence via regulation of WRKY-dependent salicylic acid accumulation and signaling. Plant Physiol. 173, 2294–2307.
Zhou, X., Jiang, Y.J., Yu, D.Q., 2011. WRKY22 transcription factor mediates dark-induced leaf senescence in *Arabidopsis*. Mol. Cells 31, 303–313.