Research paper

AtWRKY75 positively regulates age-triggered leaf senescence through gibberellin pathway

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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 may positively modulate age-triggered leaf senescence through the gibberellin-mediated signaling pathway.

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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
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′-ATATGGGCGAAAAAGCCCTGCA-3′) and WRKY75-R (5′-TGTCGCAAGTCTTCCGTTGA-3′), SAG12-F (5′-ATCCAAAAG-CACTTCTTACAGG-3′) and SAG12-R (5′-CCACTGGCCTTCCAT-CAGTCG-3′), SAG29-F (5′-GCCACCACTGGAGAAAAGG-3′) and SAG29-R (5′-CCACCAATGTGTTACCATGAA-3′), ACTIN2-F (5′-TGTGCTAATCAGGATTTT-3′) and ACTIN2-R (5′-TTGTCCCCGCTCTGCTGT-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 GA3 as described in Chen et al. (2017). Myc-WRKY75/RGL1 seedlings were harvested after treatment with 50 μM GA3 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 (At4G58890), 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)
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-senescent 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 knockdown 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
WRKY75 mutants than in WT plants (Fig. 2B). Correspondingly, both ion leakage and expression of representative senescence-induc e 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 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.
Function or RGL1 overexpression could rescue the earlier senescence phenotype caused by 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 performed using WRKY75:YFP-WRKY75:3′-WRKY75 transgenic plants. WRKY75 can directly bind to the WRKY45 promoter through the W-box cis-element (pWRKY45-2) (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.
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...
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...
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 SAGs, 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 ABF4 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; Chen et al., 2017); however, the specific mechanisms by which GA affects this progress have not yet been determined. Numerous studies have suggested that WRKY proteins function as key regulators during leaf senescence. We previously found that WRKY75 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 GA1 gain-of-function and RGL1 overexpression can partially delay the precocious senescence phenotype caused by WRKY75 overexpression (Figs. 5 and 54). Together, these data suggest that WRKY75 may function downstream of DELLA 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 del1 mutants was shortened, whereas the lifespans of the GA biosynthesis mutant ga1 and the gid1agid1agid1c 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.

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Appendix A. Supplementary data

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

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