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ORIGINAL ARTICLE

DELLA proteins negatively regulate dark-induced senescence and chlorophyll degradation in *Arabidopsis* through interaction with the transcription factor WRKY6

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Abstract

Key message **DELLA proteins' negative regulation of dark-induced senescence and chlorophyll degradation in *Arabidopsis* is through interaction with WRKY6 and thus repression of its transcriptional activities on senescence-related genes.**

Abstract Senescence is an intricate and highly orchestrated process regulated by numerous endogenous and environmental signals. Gibberellins (GAs) and their signaling components DELLA proteins have been known to participate in the regulation of senescence. However, the mechanism of the GA-DELLA system involved in the senescence process remains largely unclear. Darkness is a known environmental factor that induces plant senescence. In this study, exogenous GA_3 (an active form of GA) accelerated but paclobutrazol (a specific GA biosynthesis inhibitor) retarded dark-induced leaf yellowing in *Arabidopsis*. Moreover, the dark-triggered decrease in chlorophyll content, increase in cell membrane leakage, and upregulation of senescence-associated genes were notably impaired in both endogenous GA-decreased mutants *ga3ox1*/*ga3ox2* and *ga20ox1*/*ga20ox2* compared with those in wild-type Col-0. These effects of darkness were enhanced in the quintuple mutant of DELLA genes *gai-t6*/*rga-t2*/*rgl1-1*/*rgl2-1*/*rgl3-1* and conversely attenuated in the gain-of-function mutant *gai* and transgenic plant *35S::TAP-RGAd17* compared with wild-type Ler. Subsequently, RGA interacted with the transcription factor WRKY6 in a yeast two-hybrid assay, as confirmed by bimolecular fluorescence complementation and pull-down analyses. In addition, mutation and overexpression of *WRKY6* retarded and accelerated dark-induced senescence, respectively. Furthermore, transient expression assays in *Arabidopsis* protoplasts indicated that RGA and GAI weakened the transcriptional activities of WRKY6 on its downstream senescence-related genes, including *SAG13* and *SGR*. Taken together, these results suggest that GAs positively and DELLAs negatively regulate dark-induced senescence and chlorophyll degradation in *Arabidopsis*. DELLAs function in this process, at least in part, by interacting with WRKY6.

Keywords *Arabidopsis* · Chlorophyll degradation · Dark · DELLA · Gibberellins · Senescence

Abbreviations

BiFC	Bimolecular fluorescence complementation
della	*gai-t6*/*rga-t2*/*rgl1-1*/*rgl2-1*/*rgl3-1*
fLUC	Firefly luciferase
GA	Gibberellin
GA3ox	Gibberellin 3-oxidase
GA20ox	Gibberellin 20-oxidase
GAI	GA-insensitive
NYC1	Non-yellow coloring 1
PAC	Paclobutrazol
qRT-PCR	Real-time quantitative reverse transcription polymerase chain reaction
REN LUC	Renilla luciferase
RGA	Repressor of *ga1-3*
SGR	Stay green
SAG	Senescence-associated gene
TF	Transcription factor

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Introduction

Senescence, the final stage of plant growth and development, is not only highly regulated by a serial of internal signals including various phytohormones, reproductive development, and aging, but also induced by environmental factors, such as high or low temperature, drought, nutrient deficiency and darkness (Guo and Gan 2005; Lim et al. 2007). Senescence is characterized by chlorophyll degradation, breakdown of proteins and nucleic acids, and nutrient remobilization (Lim et al. 2007). Molecular and genetic studies have identified different sets of genes in the model plant *Arabidopsis* (*Arabidopsis thaliana*), designated as senescence-associated genes (*SAGs*) that encode proteins involved in the breakdown of chlorophyll, nucleases, proteases, and cell wall hydrolases (Lim et al. 2007; Qiu et al. 2015). Expression of *SAGs* will quickly increase upon senescence onset, and thus they are often used as molecular markers of senescence (Li et al. 2013; Sakuraba et al. 2014; Zhang et al. 2015a; Ren et al. 2017).

Loss of green color is a dramatically phenotypic change in senescing leaves, which is due to the net loss of chlorophyll in chloroplasts (Hörtensteiner 2006). A biochemical pathway has been clearly elucidated for the chlorophyll degradation in *Arabidopsis* (Hörtensteiner 2006, 2013). As the first step, chlorophyll b is converted into chlorophyll a via two reductive reactions, catalyzed by chlorophyll b reductase and 7-hydroxymethyl chlorophyll a reductase, respectively. *NON-YELLOW COLORING 1* (*NYC1*) and its homolog *NYC1-LIKE* (*NOL*) are two key genes encoding subunits of chlorophyll b reductase. In the next step, the central Mg atom and the phytol residue are removed by metal chelating substance (MCS) and pheophytin pheophorbide hydrolase (PPH), respectively, to produce pheophorbide a. The ring structure of pheophorbide a is then oxygenolytically opened by pheophorbide an oxygenase (PAO) to generate red chlcatabolite (RCC), which is further degraded by RCC reductase (RCCR) to convert to a primary fluorescent chlorophyll catabolite (*p*FCC), leading to the loss of green color (Hörtensteiner 2013; Kuai et al. 2017). Moreover, one regulator of chlorophyll breakdown, STAY-GREEN1 (SGR), was recently found to be a Mg-dechelatase (Shimoda et al. 2016), could interact with the above-mentioned chlorophyll catabolic enzymes, and is involved in destabilizing pigment-protein complexes as a prerequisite for chlorophyll degradation enzymes to access their substrates during leaf senescence (Sakuraba et al. 2012).

To illuminate senescence-associated regulatory pathway in plants, a series of functional analyses have provided many evidences showing that some WRKY transcription factors (TFs) play important roles in modulating senescence. For example, expression of *WRKY22* is enhanced by darkness treatment, and its overexpressing and mutation plants exhibit accelerated and delayed senescence phenotypes in the dark, respectively, indicating that WRKY22 plays a positive role in dark-induced senescence (Zhou et al. 2011). Interestingly, *WRKY22* is a target of another WRKY TF, WRKY53. Moreover, overexpression and RNAi of *WRKY53* accelerate and retard senescing in *Arabidopsis*, respectively (Miao et al. 2004). In addition, during leaf senescence, *WRKY53* is modulated in a direct promoter-binding manner by a single-stranded DNA-binding protein WHIRLY1 (WHY1) (Miao et al. 2013), which could be regulated by the Calcineurin B-Like-Interacting Protein Kinase14 (CIPK14) through phosphorylation (Ren et al. 2017), thus establishing a WRKY53-based regulatory pathway underlying senescence signaling in *Arabidopsis*. Loss-of-function mutant *wrky45* and overexpression plant *WRKY45OX* exhibit delayed and accelerated age-triggered leaf senescence, respectively, and consistently, expression of *SAGs* is significantly decreased and increased in *wrky45* and *WRKY45OX*, respectively, suggesting that WRKY45 is a novel positive regulator for age-mediated leaf senescence (Chen et al. 2017). In addition, WRKY6 positively influences leaf senescence through directly binding to a W-box motif in the promoter of *SENESCENCE-INDUCED RECEPTOR-LIKE* (*SIRK*) and enhancement of its transcript level (Robatzek and Somssich 2002).

Phytohormones affect plant senescence through complex interconnecting pathways, with ethylene, abscisic acid, and jasmonic acid promoting senescence, whereas cytokinin- and auxin-retarding senescence (Zhang and Zhou 2013). But only a few studies focused on gibberellins (GAs) for their potential roles on senescence regulation. Several reports consider that GAs are negative regulators for senescence, as exogenous GAs retard yellowing of detached leaves of dandelion (*Taraxacum officinale*), banana (*Musa cavendishii* Lamb.), and *Rumex* (*Rumex crispus*) (Whyte and Luckwill 1966; Goldthwaite and Laetsch 1968). However, GAs appear to play positive roles for senescence in *Arabidopsis*, as exogenous GAs accelerate age-dependent leaf senescence and such a process is retarded in GA biosynthesis mutant (Chen et al. 2014, 2017).

GAs are a large group of tetracyclic diterpene plant hormones that are essential for numerous aspects of plant growth and development, such as seed germination, stem elongation, leaf expansion, trichome development, and flowering (Davière and Achard 2013). Production of bioactive GAs from common diterpene precursor *trans*-geranylgeranyl diphosphate requires a set of enzymes, including terpene synthases, cytochrome P450 monooxygenases, and two types of 2-oxoglutarate-dependent dioxygenases: GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox) (Yamaguchi

2008). The GA signaling is received and transduced by the GID1 GA receptor/DELLA repressor pathway. GA targets DELLAs for ubiquitylation and subsequent destruction through the 26S proteasome-dependent pathway, thereby overcoming DELLA-mediated restraining effects on plant growth and development (Davière and Achard 2013). There are five DELLAs in *Arabidopsis*, namely GA-INSENSITIVE (GAI), REPRESSOR OF ga1-3 (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, which play unique and overlapping functions in repressing GA-mediated plant responses (Yamaguchi 2008; Davière and Achard 2013). DELLAs generally function through interaction with other factors. For instance, DELLAs modulate cell elongation and plant growth via interacting with phytochrome-interacting factor 3 (PIF3), a bHLH-type TF negatively involved in light-signaling transduction, and Brassinazole Resistant 1 (BZR1), a key positive TF of brassinosteroid signaling (Feng et al. 2008; Li et al. 2012); and DELLAs upregulate photoprotective enzyme Protochlorophyllide oxidoreductase (POR) at transcript level indirectly through interaction with some unidentified factors to modulate chlorophyll biosynthesis to prevent photooxidative damage during seedling deetiolation in *Arabidopsis* (Cheminant et al. 2011).

Leaf senescence occurs earlier and expression of *SAG12* and *SAG29* is enhanced in mutant *ga1-3*/*gai-t6*/*rga-t2*/*rgl1-1*/*rgl2-1* compared with the wild-type Ler, suggesting that DELLAs play negative roles in age-triggered senescence (Chen et al. 2014). Furthermore, RGL1 interacts with WRKY45 to repress its transcriptional activation on the downstream *SAGs* gene during age-triggered leaf senescence (Chen et al. 2017). Whether GA-DELLA system participates in other signaling-mediated senescence is still unclear. In this study, we showed that GAs positively and DELLAs negatively regulate dark-induced senescence in *Arabidopsis*. Moreover, RGA could physically interact with WRKY6, and the interaction results in impaired transcriptional activation of WRKY6 on the downstream *SAG* genes, including *SAG13* and *SGR*. Hence, our results provide compelling evidences that WRKY6 is a target of GA-DELLA system regulating dark-induced senescence.

Materials and methods

Plant materials and growth conditions

The mutants *ga20ox1*/*ga20ox2* and *gai* were kindly provided by Peter Hedden (Rothamsted Research, Harpenden, United Kingdom) and Nicholas P. Harberd (John Innes Centre, United Kingdom), respectively (Achard et al. 2007; Plackett et al. 2012). The transgenic plant *35S::TAP-RGAd17* (overexpressing TAP-tagged RGA but lacking a 17 amino-acid motif within the DELLA domain) was kindly provided by Xing-Wang Deng (Yale University, USA) (Feng et al. 2008). The *WRKY6* knockout mutant *wrky6-1* and *WRKY6* overexpression line *WRKY6OX* were kindly provided by Imre E. Somssich (Max-Planck-Institut, Germany) (Robatzek and Somssich 2002; Chen et al. 2009). The pentuple mutant of all five DELLA genes (*RGA, GAI, RGL1, RGL2* and *RGL3*) *gai-t6*/*rga-t2*/*rgl1-1*/*rgl2-1*/*rgl3-1* (*della*) (CS16298) and the double mutant *ga3ox1*/*ga3ox2* (CS6944) were ordered from *Arabidopsis* Biological Resource (ABRC). The *della, gai*, and *35S::TAP-RGAd17* are in Landsberg erecta (Ler) ecotype background; whereas the *ga20ox1*/*ga20ox2, ga3ox1*/*ga3ox2, wrky6-1*, and *WRKY6OX* are in Columbia-0 (Col-0) ecotype background. All *Arabidopsis* seeds used were surface sterilized with 20% (V/V) bleach solution for 10 min with gentle shaking, washed with sterile water for three times, and then sown on 0.68% phytoblend-solidified 1/2 strength Murashige and Skoog (1/2MS) media. Subsequently, the seeds were cold-treated at 4 °C for 3 days in the dark and then transferred to a chamber at 22 °C with a constant white light condition (about 80 μmol m^{-2} s^{-1}) for growth. 3 weeks later, the plants were transferred to darkness directly or after spray with 10 μM GA_3 (an active form of GAs) or 1 μM paclobutrazol (PAC, a specific GA biosynthesis inhibitor).

Measurement of chlorophyll pigments, ion leakage, malondialdehyde (MDA) content, and Evans blue staining

Total chlorophyll content, ion leakage, and MDA level of entire rosettes were determined according to Zhang et al. (2015a). The Evans blue staining was performed according to Zhou et al. (2011).

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA extraction of entire rosettes, complementary DNA synthesis, reverse transcription reaction, and quantitative PCR were carried out according to our previous report (Zhang et al. 2015a). The gene expression was normalized to *ACTIN2* to minimize variation in cDNA template levels. The primers used for qRT-PCR are listed in Supplementary Table S1.

Yeast two-hybrid (Y2H), bimolecular fluorescence complementation (BiFC), and pull-down analyses

For Y2H assay, the cDNA sequence of 387 amino acids located in the C terminus of *RGA* (*cRGA*) was PCR amplified and cloned into the *Nde*I and *Bam*HI sites of *pGBKT7* vector to generate cRGA-BD. The cDNA sequences of full-length coding sequence (CDS), 288 amino acids located

in the N terminus, and 266 amino acids located in the C terminus of *WRKY6* were individually PCR amplified and inserted into the *Nde*I and *Bam*HI sites of *pGADT7* vector to get WRKY6-AD, nWRKY-AD, and cWRKY6-AD, respectively. The resulting plasmids were introduced into yeast strain AH109 and the Y2H was carried out as described previously (Zhang et al. 2017). The primers used are listed in Supplementary Table S1.

For BiFC assay, full-length CDS of *RGA* was PCR amplified and inserted into *pUC-SPYNE* to generate a N-terminal in-frame fusion with nYFP, nYCP-RGA, while the *WRKY6* CDS was PCR amplified and introduced into *pUC-SPYCE* to generate a N-terminal in-frame fusion with cYFP, cYFP-WRKY6, using the ClonExpress® II One Step Cloning Kit (Vazyme Biotech Co., Ltd) following the manuscript's protocol. The primers used for cloning were listed in the Supplementary Table S1. The *Arabidopsis* mesophyll protoplasts were prepared from 4-weeks-old Col-0 seedlings grown under short photoperiod as described previously (Wu et al. 2009), and the subsequent PEG transfections were carried out as described by Yoo et al. (2007). YFP fluorescence was detected under a confocal laser scanning microscope (Leica Microsystems) after 12–18 h transfection.

For pull-down assay, *cRGA* was cloned into *pGEX-4T-1* to get cRGA-GST, while the full-length *WRKY6* CDS and *cWRKY6* were inserted into *pET-32a* to get WRKY6-6×His and cWRKY6-6×His, respectively. cRGA-GST and the empty vector were transferred individually into *Escherichia coli* BL21, induced by IPTG, and purified by glutathione agarose resin (Thermo Scientific); whereas WRKY6-6×His, cWRKY6-6×His and *pET-32a* were transferred individually into *Escherichia coli* BL21 (DE3), induced by IPTG, and purified with Capturem™ 6×His-Tagged Purification Miniprep Kit (Clontech). The pull-down analyses were performed as described previously (Oh et al. 2012). In brief, 5 μg of cRGA-GST-bound glutathione agarose beads were incubated with either 2 μg of TrxA-6×His or WRKY6-6×His or cWRKY6-6×His in 1×PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH7.4) at 4 °C on an end-over-end rocking platform for 1 h. The beads were then washed three times with 1×PBS buffer. Discard the washes, and directly add an equal volume of 2×SDS gel-loading buffer to the beads, which were then subsequently analyzed by western blot using anti-6×His antibody (BBI) at 1:10,000 dilution. The western blot assays were carried out as described previously (Zhang et al. 2015b).

Transient transcription expression assays in *Arabidopsis* protoplasts

The transient transcription expression assays were performed as described previously (Zhang et al. 2017). The full-length CDSs of *RGA, GAI*, and *WRKY6* were cloned into *pGreenII 62-SK* to get the effectors, and about 2 kb length of promoters of *SAG13* and *SGR* that were amplified from genomic DNA of Col-0 were introduced into *pGreenII 0800-LUC* to get the reporters. All primers used can be found in the Supplementary Table S1. *Arabidopsis* mesophyll protoplasts were prepared from 4-weeks-old Ler, *della, gai*, and *35S::TAP-RGAd17* seedlings grown under short photoperiod using tape method (Wu et al. 2009), and subsequent transfections were performed following the protocol as described previously (Yoo et al. 2007). Firefly luciferase (fLUC) and renilla luciferase (REN LUC) activities were determined with the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

The significance of differences between datasets were evaluated using paired student's *t* test using the originPro8.0 software (OriginLab).

Accession numbers

Sequence data from this study can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *ACTIN2* (At3g18780); *SAG13* (At2g29350), *SAG113* (At5g59220), *SGR* (At4g22920), *NYC1* (At4g13250), *GAI* (At1g14920); *RGA* (At2g01570); *WRKY6* (At1g19670).

Results

GAs positively regulate dark-induced senescence and chlorophyll degradation in *Arabidopsis*

Recent reports have shown that GAs exhibit positive effects on age-triggered senescence in *Arabidopsis* (Chen et al. 2014, 2017); thus, we were interested to determine whether GAs are involved in dark-induced senescence as well. After 3-weeks-old Col-0 seedlings were transferred to darkness for 4 days, the GA_3-treated seedlings had less chlorophyll content and more cell membrane damage, whereas PAC-treated seedlings had significantly higher chlorophyll content and less ion leakage compared with the control (Fig. 1). Thus, exogenous GAs positively regulated dark-induced senescence and chlorophyll degradation. To strengthen this notion, 3-weeks-old Col-0 together with *ga30×1/ga3ox2* and *ga20ox1/ga20ox2*, which are two mutants with low endogenous GA levels (Mitchum et al. 2006; Plackett et al. 2012), were transferred to darkness. As results, *ga30×1/ga3ox2* and *ga20ox1/ga20ox2* were markedly greener compared to their wild-type Col-0 after 4 days (Fig. 2a). A detailed time-course experiment was subsequently carried out to determine the changes in chlorophyll

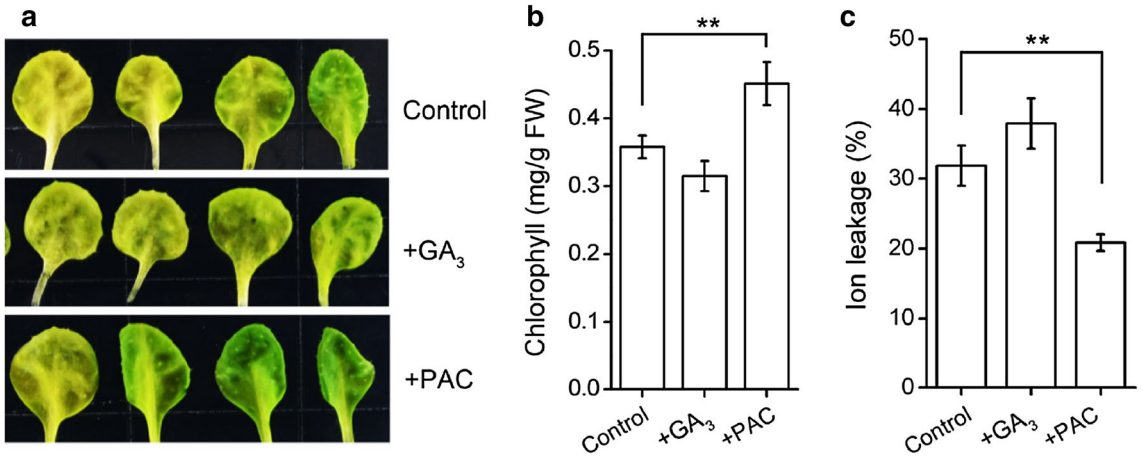

Fig. 1 Effect of exogenous GA_3 and PAC on dark-induced senescence. **a** The third to sixth true leaves of each representative 3-weeks-old light-grown Col-0 seedling transferred to darkness for 4 days with treatment of 10 μM GA_3 (+GA_3), 1 μM PAC (+PAC), or nothing (Control). Chlorophyll levels (**b**) and ion leakage (**c**) were determined for above plants (**a**). Error bars indicate the SE based on three biological replicates, and values are means ± SE. Double asterisks represent the significant difference between Control and PAC-treatment at the level of $P < 0.01$ based on Student's *t* test

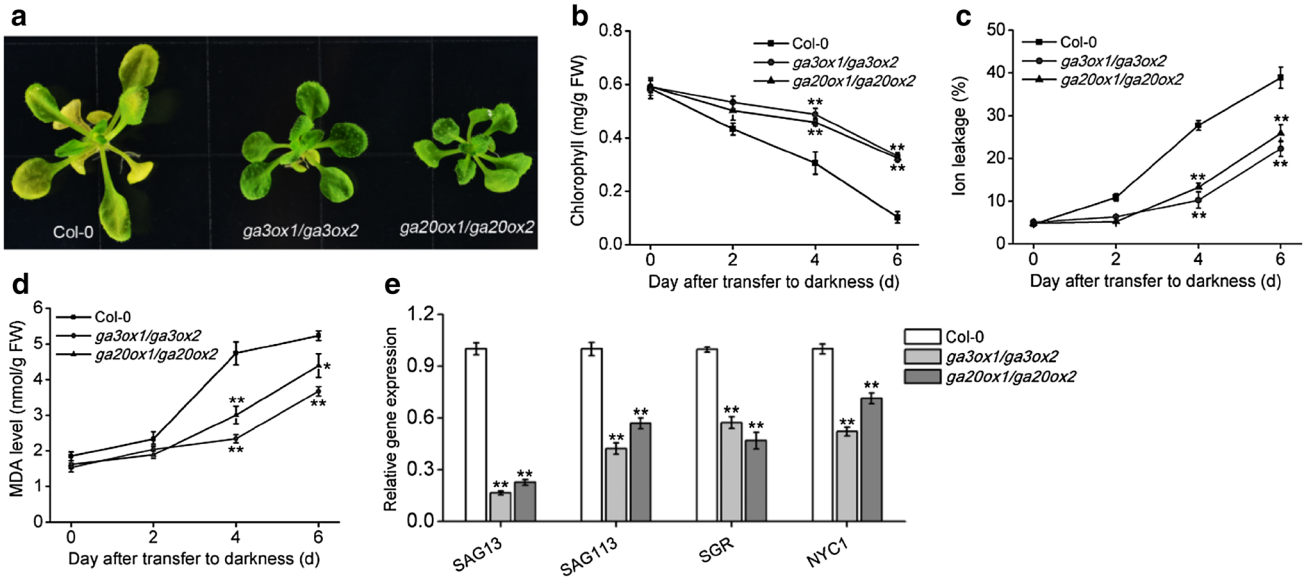

Fig. 2 Dark-induced senescence is impaired in both endogenous GAs-decreased mutants. **a** Representative seedlings of 3-weeks-old light-grown mutants *ga3ox1/ga3ox2* and *ga20ox1/ga20ox2*, as well as the wild-type Col-0 transferred to darkness for 4 days. Chlorophyll content (**b**), Ion leakage (**c**), and MDA level (**d**) were determined after 3-weeks-old light-grown plants of Col-0, *ga3ox1/ga3ox2*, and *ga20ox1/ga20ox2* were transferred to darkness for 0, 2, 4, 6 d. **e** Transcript levels of *SAG13, SAG113, SGR*, and *NYC1* were determined after 3-weeks-old light-grown indicated plants transferred to darkness for 4 days. Error bars indicate the SE based on three biological replicates, and values are means ± SE. * and ** represent significance of differences at the levels of $P < 0.05$ and $P < 0.01$ compared to the data of Col-0, respectively, based on the Student's *t* test

levels, ion leakage, and MDA content. The results showed that as dark time was extended, chlorophyll levels decreased in all seedlings tested, but such a decrease was much slower in *ga30×1/ga3ox2* and *ga20ox1/ga20ox2* than in Col-0 (Fig. 2b). Consistently, after the same treatment, ion leakage and the MDA level increased slower in *ga30×1/ga3ox2* and *ga20ox1/ga20ox2* than those in Col-0 (Fig. 2c, d). To further elucidate the role of GAs during senescence, expression of both *SAGs* (*SAG13* and *SAG113*) and both chlorophyll degradation-related genes (*SGR* and *NYC1*) were tested. *SAG13, SAG113, SGR*, and *NYC1* expression were significantly lower in *ga30×1/ga3ox2* and *ga20ox1/ga20ox2* compared

to Col-0 after 4 days of darkness treatment of 3-weeks-old seedlings (Fig. 2e). These data collectively indicate that decreases in endogenous GAs will retard dark-triggered senescence and chlorophyll degradation in *Arabidopsis*.

DELLAs are negative regulators for dark-induced senescence in *Arabidopsis*

Given that DELLAs are important components involved in GA signaling, we next asked if DELLAs were responsible for the negative regulation of GAs during dark-induced senescence. Therefore, the effects of dark-induced senescence were determined in mutant *della*, GA-insensitive mutant *gai*, transgenic line *35S::TAP-RGAd17*, and their wild-type Ler. As shown in Fig. 3a, the *della* mutant was yellower, whereas *gai* and *35S::TAP-RGAd17* was greener compared to Ler after plants were transferred to darkness for 3 or 4 days. All tested seedlings had similar chlorophyll content and ion leakage before the darkness treatment (Fig. S1). However, chlorophyll content was markedly lower in *della* after 3 or 4 days of darkness treatment, but significantly more chlorophyll was found in *gai* and *35S::TAP-RGAd17* compared to Ler (Fig. 3b). Consistently, ion leakage was higher in *della*, but lower in *gai* and *35S::TAP-RGAd17* than in Ler (Fig. 3c). In addition, Ler had more dead cells than *gai* and *35S::TAP-RGAd17* after 4 days of darkness treatment, as characterized by Evans Blue staining (Fig. 3d). These results indicate that DELLAs, at least RGA and GAI, are negative regulators of dark-induced senescence and chlorophyll degradation. To clarify whether DELLAs regulate dark-induced senescence at the transcriptional level, we also tested *SAG13, SAG113, SGR*, and *NYC1* transcript levels at 0, 2, and 4 days after the darkness treatment. The results showed that expression of these genes increased as seedlings were transferred to darkness (Fig. 3e). Such an increase was faster in *della* but slower in *gai* and *35S::TAP-RGAd17* compared to Ler (Fig. 3e), indicating that DELLAs are negatively involved in darkness upregulation of *SAGs* and chlorophyll degradation-related genes.

RGA physically interacts with WRKY6 *in vivo* and *in vitro*

We asked how DELLAs participate in dark-induced senescence and chlorophyll degradation. As DELLAs are involved in the regulation of many processes often through direct

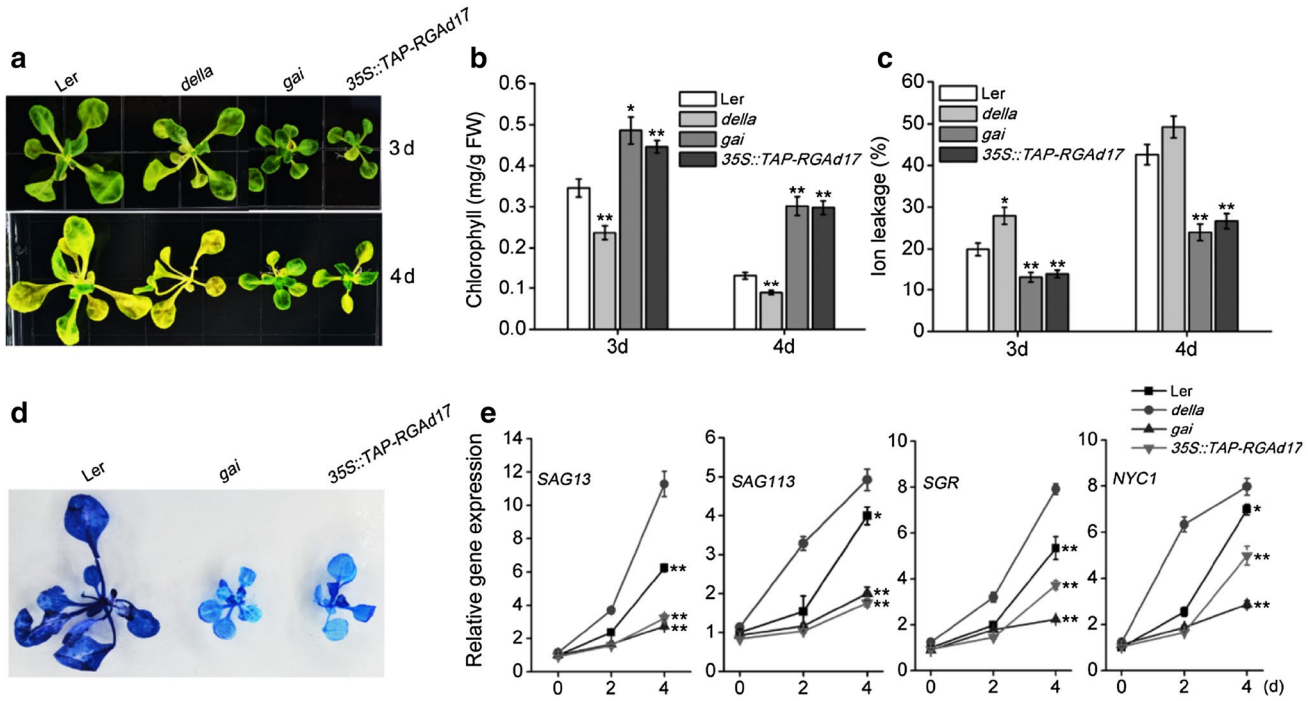

Fig. 3 DELLA proteins positively regulate dark-induced senescence. **a** Representative seedlings of 3-weeks-old light-grown mutant *della* and *gai*, transgenic plant *35S::TAP-RGAd17*, and their wild-type Ler transferred to darkness for 3 or 4 days. Chlorophyll content (**b**) and Ion leakage (**c**) were determined after 3-weeks-old light-grown plants of Ler, *della, gai*, and *35S::TAP-RGAd17* were transferred to darkness for 3 and 4 days. **d** Represent seedlings of 3-weeks-old light-grown Ler, *gai*, and transgenic plant *35S::TAP-RGAd17* that were analyzed using Evans blue staining after transfer to darkness for 4 days. **e** Transcript levels of *SAG13, SAG113, SGR*, and *NYC1* were determined after 3-weeks-old light-grown indicated plants transferred to darkness for 4 days. Error bars indicate the SE based on three biological replicates, and values are means ± SE. * and ** represent significance of differences at the levels of $P < 0.05$ and $P < 0.01$ compared to the data of Ler, respectively, based on the Student's *t* test

interactions with other TFs, such as PIF3 and BZR1, we subsequently tested whether relationships exist between DELLAs and some known WRKY TFs that are involved in senescence. Thus, Y2H assays were carried out. As the full-length DELLA genes (*RGA, GAI, RGL1, RGL2*, and *RGL3*) all displayed strong auto-activation when fused with the GAL4 DNA-binding domain, the truncated C-terminal part of RGA (cRGA), which only exhibited slight auto-activation, was subsequently used for the Y2H assay. Interestingly, we identified that cRGA strongly interacted with the full-length WRKY6 and the C-terminal part of WRKY6 (cWRKY6) but not the N-terminal of WRKY6 (nWRKY6) (Fig. 4a). To further confirm this interaction, BiFC and pull-down assays were subsequently carried out. As shown in Fig. 4b, YFP fluorescence was only detected when nYFP-RGA and cYFP-WRKY6 were co-transfected into *Arabidopsis* protoplasts but no yellow fluorescence was detected when nYFP-RGA together with the empty vector cYFP, or the empty vector nYFP together with cYFP-WRKY6 were co-transfected into *Arabidopsis* protoplasts, indicating an interaction between RGA and WRKY6 in vivo. As shown in Fig. 4c, the TrxA-6×His-tagged proteins WRKY6 and cWRKY6 but not TrxA-6×His alone were pulled down by GST-tagged cRGA. Taken together, these results indicate that RGA interacts with WRKY6 in vivo and in vitro.

WRKY6 positively regulates dark-induced senescence and chlorophyll degradation

Next, we wondered whether WRKY6 participates in dark-induced senescence and chlorophyll degradation. To this end, 3-weeks-old mutant *wrky6-1*, overexpressing line *WRKY6OX*, and wild-type Col-0 plants were transferred to darkness. After 4 days, *wrky6-1* was greener and *WRKY6OX*

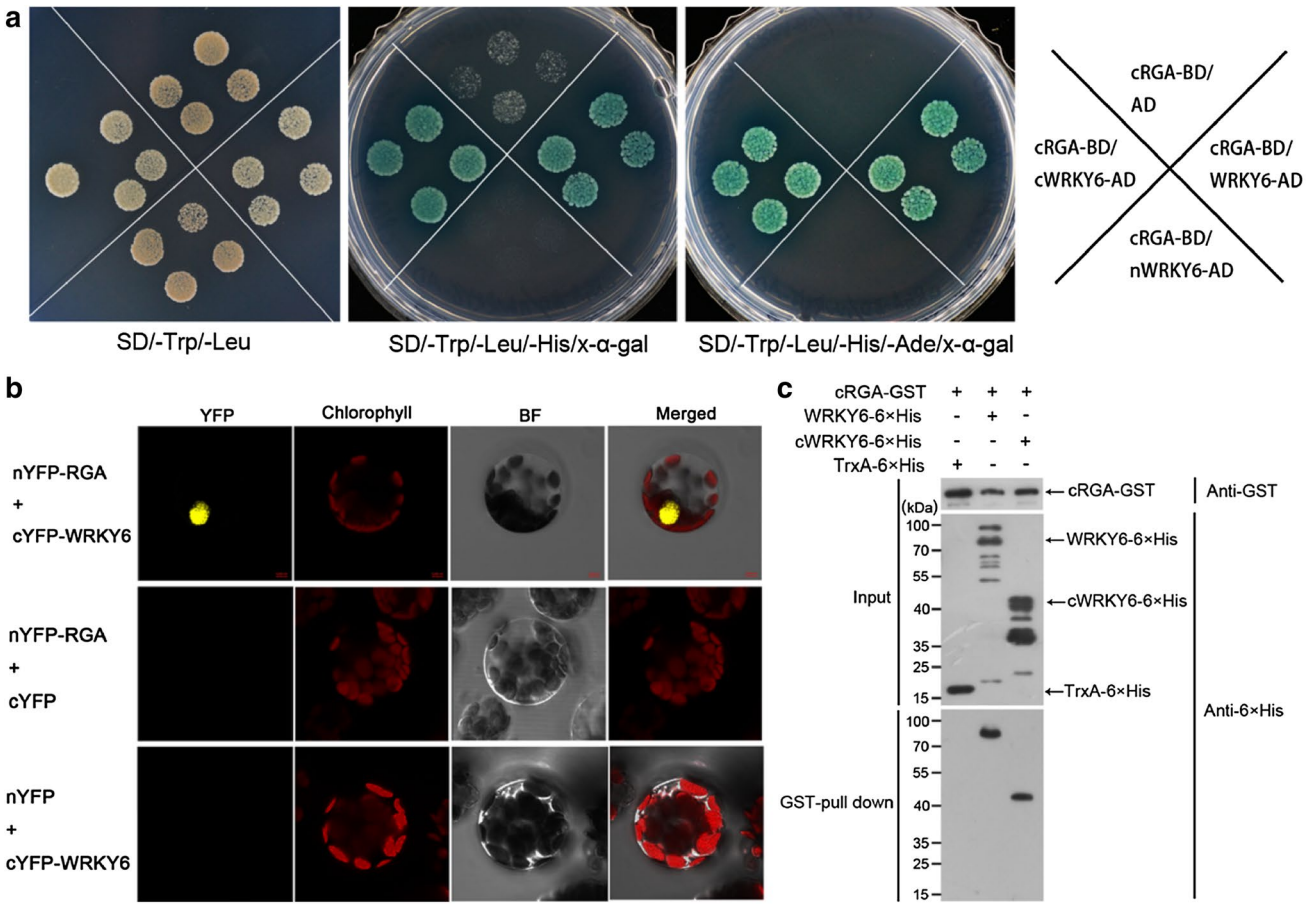

Fig. 4 RGA interacts with WRKY6. **a** Yeast two-hybrid assays to detect the interactions of C-terminal of RGA (cRGA) with full-length of WRKY6, N-terminal of WRKY6 (nWRKY6), and C-terminal of WRKY6 (cWRKY6) in yeast AH109, and the relationship between cRGA and AD was used as negative control. BD, pGBKT7; AD, pGADT7. **b** BiFC analysis confirmation of the interaction between RGA and WRKY6 in *Arabidopsis* protoplasts. YFP, yellow fluorescence protein; nYFP, N-terminal of YFP; cYFP, C-terminal of YFP; BF, bright field. **c** Pull-down assay of GST-tagged RGA expressed in *Escherichia coli* BL21 with 6×His-tagged WRKY6 or cWRKY6 expressed in *Escherichia coli* BL21 (DE3). Purified proteins were pulled down by glutathione resin and detected using an anti-6×His antibody. Each experiment was repeated three times with similar results and the representative results are displayed here

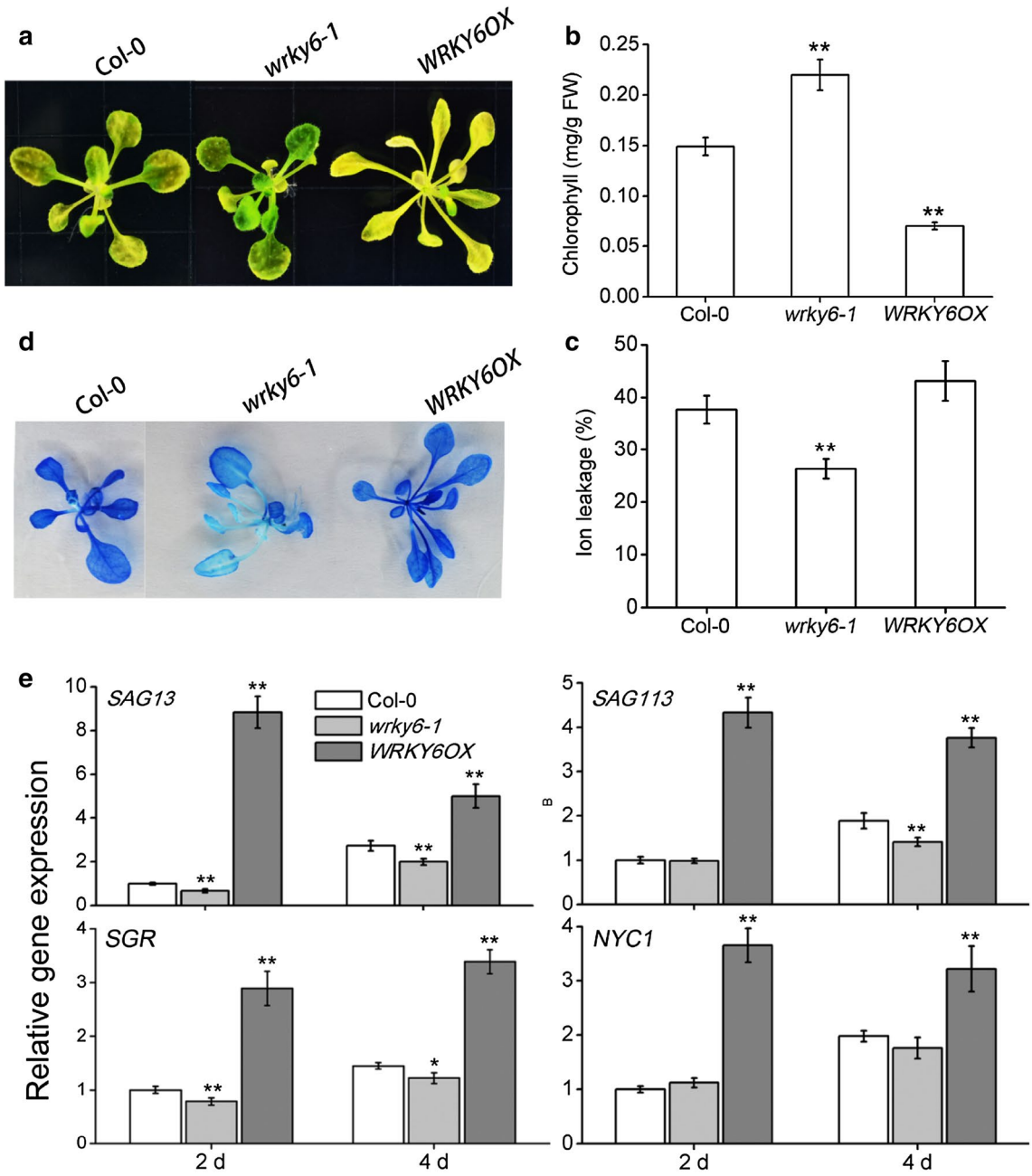

Fig. 5 WRKY6 is a positive regulator for dark-induced senescence in *Arabidopsis*. **a** Representative seedlings of 3-weeks-old light-grown *wrky6-1, WRKY6OX*, and their wild-type Col-0 that were transferred to darkness for 4 d. Chlorophyll content (**b**) and Ion leakage (**c**) were determined in the seedlings (**a**). **d** Represent seedlings of 3-weeks-old light-grown Col-0, *wrky6-1*, and *WRKY6OX* that were analyzed using Evans blue staining after transfer to darkness for 4 days. **e** Transcript levels of *SAG13, SAG113, SGR*, and *NYC1* were determined after 3-weeks-old light-grown indicated plants transferred to darkness for 4 days. Error bars indicate the SE based on three biological replicates, and values are means ± SE. * and ** represent significance of differences at the levels of $P < 0.05$ and $P < 0.01$, respectively, based on the Student's *t* test

became much yellower compared to that of Col-0 (Fig. 5a). Chlorophyll content was consistently significantly higher in *wrky6-1* after the same treatment, but markedly lower in *WRKY6OX* than that in Col-0 (Fig. 5b). Ion leakage was significantly lower in *wrky6-1* and slightly higher in *WRKY6OX* compared to that in Col-0 (Fig. 5c). Furthermore, the cell death ratio characterized by Evens Blue staining was significantly lower in *wrky6-1* than that in Col-0 (Fig. 5d). These physiological data collectively indicate that WRKY6 positively regulate dark-induced senescence and chlorophyll

degradation. Expression of the *SAG13, SGA113, SGR*, and *NYC1* genes was determined by qRT-PCR after 3-weeks-old seedlings were transferred to darkness for 2 and 4 days. As shown in Fig. 5e, expression of these four genes was significantly higher in *WRKY6OX* than that in Col-0, and expression of *SAG13* and *SGR* were markedly lower in *wrky6-1* than in Col-0, indicating that WRKY6 is a positive regulator of these four genes, particularly *SAG13* and *SGR*.

RGA negatively regulates WRKY6 upregulation of senescence-related gene expression

The interaction between RGA and WRKY6 prompted us to determine whether RGA affects transcriptional activities of WRKY6 on its potential senescence-associated genes, such as *SAG13* and *SGR*. Transient transcriptional expression assays were carried out in *Arabidopsis* protoplasts. We individually introduced full-length CDSs of *RGA, GAI*, and *WRKY6* into the *pGreenII 62-SK* vector to obtain the effectors, and introduced the *SAG13* and *SGR* promoters into the *pGreenII 0800-LUC* to obtain the reporters (Fig. 6a). The fLUC activities remarkably increased after transformation of WRKY6 together with the reporters into Ler, *della, gai*, and *35S::TAP-RGAd17* protoplasts compared to those transformed with the empty vector, indicating that WRKY6 positively regulates *SAG13* and *SGR* promoter activities (Fig. 6b). Alternatively, it should be noted that these effects were significantly enhanced in the *della* mutant but notably or slightly decreased in *gai* and *35S::TAP-RGAd17* compared to those in Ler (Fig. 6b). More importantly, applying either RGA or GAI significantly or slightly impaired the effects of WRKY6 on fLUC activities in Ler (Fig. 6b). Taken together, these results indicate that both RGA and GAI can repress WRKY6 transcriptional activities on *SAG13* and *SGR*.

Discussion

Senescence in plants can be induced or suppressed by various phytohormones. Many studies have clearly showed that ethylene, abscisic acid, and jasmonic acid promote leaf senescence, whereas cytokinins and auxin repress leaf senescence (Zhang and Zhou 2013). This appears contradictory for the potential effects of GAs on leaf senescence. Several studies have reported that GAs are effective in retarding senescence and exogenously applied GAs repress yellowing of detached leaves in dandelion,

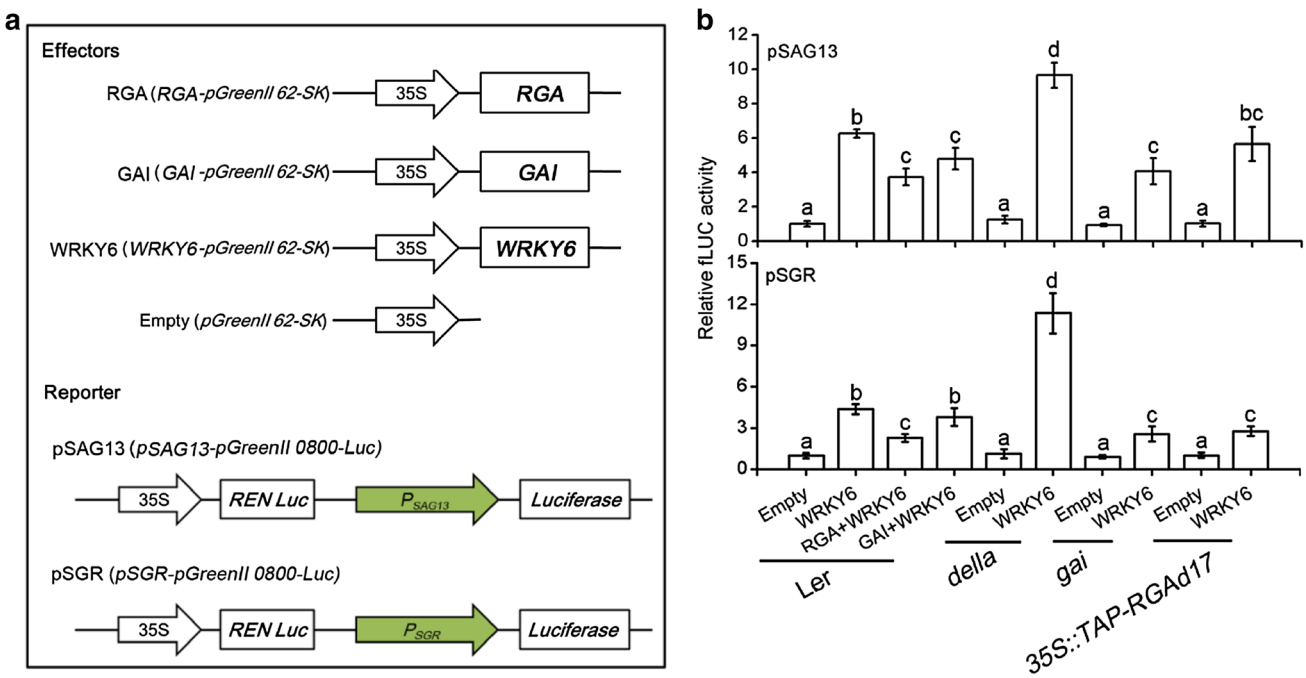

Fig. 6 RGA and GAI could impair the transcriptional activities of promoters of *SAG13* and *SGR* in *Arabidopsis* protoplasts. **a** Schematic maps of the effector and reporter constructs used in protoplast-transient expression assays. RGA, GAI, and WRKY6 represent constructs with full-length CDSs of the corresponding genes introduced into vector *pGreenII 62-SK*; Empty represent the empty vector; pSAG13 and pSGR represent the constructs with corresponding promoter sequences in the vector *pGreenII 0800-LUC*; REN Luc, renilla luciferase. **b** Relative firefly luciferase (fLUC) activities determined after the constructs were transferred into protoplasts as indicated and then cultured overnight (about 12 h). Each value is the mean of three biological replicates ± SE. Different lowercase letters on each bar represent significance of differences at the level of $P < 0.05$ according to the Student's *t* test

banana, and *Rumex* (Whyte and Luckwill 1966; Goldthwaite and Laetsch 1968). However, one study showed that exogenously applied GA_3 to *Arabidopsis* rosette leaves promotes leaf senescence, and leaves of *ga1-3*, a mutant whose endogenous GA biosynthesis is blocked, are markedly greener than those of wild-type during age-triggered senescence (Chen et al. 2014), indicating that GAs are inducers of age-mediated senescence in *Arabidopsis*. This notion was soon supported by another study, reporting that an exogenous spray of 500 μM GA_3 onto 8-days-old *Arabidopsis* seedlings every other day sped up leaf yellowing (Chen et al. 2017). Moreover, our present study also demonstrates that applying GA_3 promoted, whereas PAC notably retarded, dark-induced senescence and chlorophyll degradation in *Arabidopsis* (Fig. 1). Furthermore, the endogenous low GA mutants *ga20ox1*/*ga20ox2* and *ga3ox1*/*ga3ox2* exhibited delayed dark-induced senescence phenotypes and decreased *SAG* gene expression compared to the wild-type (Fig. 2). Hence, together with the findings by Chen et al. (2014, 2017), our results demonstrate that GAs are positive regulators of senescence and chlorophyll degradation. These inconsistent roles of GAs in leaf senescence are likely due to differences in plant species and treatment methods used in the experiments. More experiments are warranted in a future study to illuminate the exact roles underlying GA-mediated senescence.

DELLAs participate in almost all known GA-mediated processes, including seed germination and plant growth (Davière and Achard 2013). We found here that the loss-of-function mutant *della* became senescent much earlier, whereas the gain-of-function mutant *gai* and transgenic plant *35S::TAP-RGAd17* exhibited delayed yellowing phenotypes after the darkness treatment compared to the wild-type, suggesting that DELLAs are negative regulators of dark-induced senescence. Similarly, Chen et al. (2014) reported that age-dependent leaf senescence occurs earlier after DELLAs are partially removed; Chen et al. (2017) further showed that age-triggered leaf senescence is markedly accelerated in the *della* mutant compared with wild-type, whereas overexpression of the DELLA protein RGL1 dramatically conferred enhanced leaf longevity. Taken together, we conclude that GAs modulate age-triggered and dark-induced senescence through DELLAs. However, it should be noted that a DELLA-independent pathway also exists in plants to regulate GA responses. For example, the quadruple DELLA mutant *ga1-3*/*gai-t6*/*rga-t2*/*rgl1-1*/*rgl2-1* is cytokinin-sensitive, but exogenous GAs suppress various cytokinin responses in this mutant, suggesting that GAs regulate cytokinin responses via a DELLA-independent pathway (Maymon et al. 2009). Therefore, we cannot completely exclude the possibility that the regulation of plant senescence by GAs occurs partially through a DELLA-independent pathway.

As DELLAs do not contain any recognizable DNA-interacting domain, they are unlikely to regulate the downstream target genes through directly binding to the promoters, but instead, they act in association with other transcription factors (Davière and Achard 2013; Xu et al. 2014). For example, DELLAs interact with PIF3 and prevent PIF3 from binding to its downstream target gene promoters; thus, abrogate PIF3-mediated light control of hypocotyl elongation (Feng et al. 2008); RGA interacts with BZR1 to block its transcriptional activities on target genes related to cell growth (Li et al. 2012). Similarly, we found here that RGA interacted with WRKY6 during regulation of senescence (Figs. 4, 5). Furthermore, transient expression assays showed that the interaction impaired transcriptional activities of WRKY6 on its downstream senescence-related gene expression including *SAG13* and *SGR* (Fig. 6). Alternatively, the effects of WRKY6 were comparably impaired in *gai* and *35S::TAP-RGAd17* compared to that in wild-type (Fig. 6), indicating that RGA and GAI may play overlapping roles during regulation of senescence by repressing WRKY6 function. Whether other DELLAs (RGL1, RGL2 and RGL3) play the same role during this process requires further elucidation. RGL1 interacts with another WRKY transcription factor, WRKY45, which is a positive regulator involved in age-dependent senescence, and such an interaction leads to impaired transcriptional activities on WRKY45 target genes including *SAG12* and *SAG113* (Chen et al. 2017). However, whether the interaction between RGA and WRKY6, as observed in our study, contributes to age-triggered or other factor-mediated senescence, or an interaction between RGL1 and WRKY45, as established by Chen et al. (2017), could also contribute to dark-induced or other factor-mediated senescence, remain unclear.

WRKY6 was involved in senescence based on the finding that senescing leaves of *wrky6* knockout mutants decreased expression of *Senescence-Induced Receptor-like Kinase*(*SIRK*), but green leaves of *WRKY6* overexpression lines showed increased expression of *SIRK*, a gene encoding a receptor-like protein kinase, whose developmental expression is strongly induced specifically during age-triggered senescence (Robatzek and Somssich 2002). Moreover, WRKY6 specifically activates *SIRK* transcription by directly binding to the W-box motif (TGACC/T) located in the *SIRK* promoter (Robatzek and Somssich 2002). Interestingly, we observed that dark-induced senescence was retarded in the *wkry6-1* knockout mutant but was accelerated in overexpression plant *WRKY6OX* (Fig. 5). Furthermore, we found previously that expression of *WRKY6* increases as darkness is extended (Zhang et al. 2015a). Therefore, we conclude that WRKY6 is a positive regulator involved in dark-induced senescence. The qRT-PCR assay revealed that the expression of several senescence-related genes, especially *SAG13* and *SGR*, was significantly lower after the darkness treatment in

wrky6-1 but significantly higher in *WRKY6OX* compared to that in the wild-type (Fig. 5e). WRKY6 notably enhanced *SAG13* and *SGR* transcription in the protoplasts tested (Fig. 6). These data at least partially suggest that *SAG13* and *SGR* are downstream targets of WRKY6 during modulation of senescence. However, more experiments are required to determine whether WRKY6 directly binds to the W-box motifs in the promoters of these genes or indirectly increases expression of these gene by interacting with other transcription factors.

We previously identified PIF5 as a key factor that positively regulates dark-induced senescence upstream of *ORE1* and modulates chlorophyll breakdown upstream of *SGR* and *NYC1* (Zhang et al. 2015a). We wondered whether some relationship exists between GA-DELLA signaling and the PIF5 pathway during regulation of dark-induced senescence. However, upregulation of *WRKY6* by darkness was not significantly affected by a *PIF5* mutation or overexpression (Zhang et al. 2015a). Moreover, no interactions were observed between PIF5 and DELLAs/WRKY6 in our experiments (data not shown). However, darkness increases endogenous GA content in *Arabidopsis* by regulating several GA biosynthetic genes including *GA20ox1* and *GA3ox1* at the transcriptional level, which, in turn, may decrease DELLA accumulation (Fig. S2; Achard et al. 2007). We propose a model for how darkness induces senescence: *WRKY6* expression increases but that of *DELLAs* decreases when plants are transferred to darkness; thus, the repression of DELLAs on WRKY6 transcriptional activities is attenuated, and the expression of downstream senescence-related genes including *SAG13* and *SGR* increased, which initiates senescence.

Taken together, we conclude that DELLAs negatively regulate dark-induced senescence and chlorophyll degradation, at least in part, through a physical interaction with WRKY6 and repression of its transcriptional activities on senescence-related genes at the molecular level.

Author contribution statement The experiments were conceived and designed by YZ, ZL and WL. The experiments were performed by YZ, ZL, XW, JW, Kf, and ZL. The data were analyzed by YZ, ZL and WL. YZ, ZL and WL wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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