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Arabidopsis FAR-RED ELONGATED HYPOCOTYL3 Integrates Age and Light Signals to Negatively Regulate Leaf Senescence

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Leaf senescence is tightly regulated by numerous internal cues and external environmental signals. The process of leaf senescence is promoted by a low ratio of red to far-red (R:FR) light, FR light, or extended darkness and is repressed by a high ratio of R:FR light or R light. However, the precise regulatory mechanisms by which plants assess external light signals and their internal cues to initiate and control the process of leaf senescence remain largely unknown. In this study, we discovered that the light-signaling protein FAR-RED ELONGATED HYPOCOTYL3 (FHY3) negatively regulates age-induced and light-mediated leaf senescence in Arabidopsis (*Arabidopsis thaliana*). FHY3 directly binds to the promoter region of transcription factor gene *WRKY28* to repress its expression, thus negatively regulating salicylic acid biosynthesis and senescence. Both the *fhy3* loss-of-function mutant and *WRKY28*-overexpressing Arabidopsis plants exhibited early senescence under high R:FR light conditions, indicating that the FHY3–*WRKY28* transcriptional module specifically prevents leaf senescence under high R:FR light conditions. This study reveals the physiological and molecular functions of FHY3 and WRKY28 in leaf senescence and provides insight into the regulatory mechanism by which plants integrate dynamic environmental light signals and internal cues to initiate and control leaf senescence.

INTRODUCTION

Senescence, the final stage of leaf development, is tightly controlled by numerous internal cues (including age, development, sugar, and nitrogen levels) and external environmental factors (including light, temperature, and stress). In annual plants such as Arabidopsis (*Arabidopsis thaliana*) and many crops, leaf senescence indicates that the whole plant will soon die and allows the remobilization of nutrients into growing organs, such as young leaves or storage organs (Schippers et al., 2015; Woo et al., 2019); therefore, leaf senescence is critical for plant fitness and crop productivity (Gregersen et al., 2013; Bengoa Luoni et al., 2019). In general, senescence is age-triggered and initiates from the tip of the mature leaf (Woo et al., 2019). At the adult stage, accelerated or precocious leaf senescence can be observed in plants grown under canopy shade conditions, low light intensity, or after an extended darkness treatment. By contrast, a high ratio of red (R, 660 nm) to far-red (FR, 730 nm) light (indicated as R:FR), R light, or high light intensity can inhibit this process, indicating that light signals play essential roles in regulating leaf senescence (Brouwer et al., 2012; Sakuraba et al., 2014; Song et al., 2014; Liebsch and Keech, 2016; Lim et al., 2018).

The R:FR ratio of sunlight, an important environmental signal, is ~1.2 above the canopy of leaves and decreases to as low as 0.1 (a low R:FR ratio) underneath the canopy (Franklin, 2008; Casal, 2013). In most plant species such as Arabidopsis and maize (*Zea mays*), canopy shade (low R:FR) growth conditions promote cell elongation in hypocotyls, stems, and petioles, allowing the plant to capture more unfiltered light (Franklin, 2008; Kami et al., 2010; Fraser et al., 2016; Ma and Li, 2019; Shi et al., 2019). Prolonged canopy shade induces early flowering and precocious leaf senescence, subsequently affecting seed production and plant survival (Franklin, 2008; Brouwer et al., 2012; Casal, 2013). Arabidopsis phytochrome B (phyB), the major photoreceptor of R light, inhibits extended dark-induced precocious leaf senescence largely by promoting the degradation of PHYTOCHROME INTERACTING FACTOR4 (PIF4) and PIF5, two transcription factors that positively regulate leaf senescence in shade (a low R:FR ratio) or extended darkness (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015; Lim et al., 2018). However, the regulatory mechanisms by which a high R:FR light signal represses senescence remain largely unknown.

Arabidopsis FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and its closest homolog FAR-RED IMPAIRED RESPONSE1 (FAR1) are transposase-derived transcription factors that play key roles in the phytochrome A (phyA)–mediated FR light signaling pathway (Hudson et al., 1999; Wang and Deng, 2002; Lin et al., 2007). Recent studies have implicated FHY3 and FAR1 in multiple cellular processes involved in growth and development, such as the shade avoidance response, regulation of the circadian clock, flowering time regulation, chlorophyll biosynthesis, chloroplast development, abscisic acid (ABA) signaling, oxidative stress and cell death, floral development, inositol biosynthesis, starch biosynthesis, and phosphorus uptake (Li et al., 2011, 2016; Wang and Wang, 2015; Ma et al., 2016; Wang et al., 2016; Liu et al., 2017,

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2019; Ma et al., 2017, 2019; Ma and Li, 2018). Chromatin immunoprecipitation sequencing (ChIP-seq) analyses have identified over 1000 putative direct targets of FHY3, further suggesting it plays key roles in plant growth and development (Ouyang et al., 2011; Li et al., 2016); however, the physiological and molecular roles of FHY3 in leaf senescence remain unknown.

The physiological and molecular roles of various phytohormones in leaf senescence have been intensively studied. In general, cytokinin, gibberellic acid, and auxin repress leaf senescence, whereas ethylene, ABA, salicylic acid (SA), and jasmonic acid (JA) promote this process (Jibran et al., 2013; Schippers et al., 2015). After senescence initiation, the SA content of leaves continually increases, accompanied by high expression levels of many pathogenesis-related (PR) genes such as *PR1*, *PR2*, and *PR5* (Morris et al., 2000). Reducing the SA content by disrupting its key biosynthesis gene *SA INDUCTION DEFICIENT2/ISOCHORISMATE SYNTHASE1* (*SID2*/*ICS1*) or blocking the functions of its signal transduction components completely or largely rescued precocious senescence caused by starvation, disease, or stress in Arabidopsis (Guo et al., 2017; Zhang et al., 2017). By contrast, increasing SA accumulation by disrupting *SA-3-HYDROXYLASE* (*S3H*) resulted in precocious leaf senescence in Arabidopsis (Zhang et al., 2013). However, how SA biosynthesis is regulated during leaf senescence remains largely unknown.

At the molecular level, several well-studied *SENESCENCE-ASSOCIATED GENES* (*SAGs*), such as *SAG12*, *SAG13*, *SAG29*, *SAG101*, and *SAG113*, are strongly expressed in senescing leaves (Nam, 1997; Buchanan-Wollaston et al., 2005; Guo and Gan, 2005). In addition, multiple types of leaf senescence-related transcription factor genes, such as NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) and WRKY family members, are highly expressed in senescing leaves. These transcription factors control leaf senescence by regulating the transcription of different targets, including a large set of *SAGs* (Buchanan-Wollaston et al., 2005). In Arabidopsis, over 18 *WRKY* genes are highly expressed in senescing leaves (Robatzek and Somssich, 2001; Breeze et al., 2011; Zentgraf and Doll, 2019). Among these transcription factors, WRKY6, WRKY22, WRKY45, WRKY53, and WRKY75 positively regulate leaf senescence while WRKY57, WRKY54, and WRKY70 negatively regulate this process (Robatzek and Somssich, 2002; Miao et al., 2004; Zhou et al., 2011; Besseau et al., 2012; Jiang et al., 2014; Guo et al., 2017; Zentgraf and Doll, 2019). Although multiple WRKY transcription factors have been identified as key players in the regulation of leaf senescence, the regulatory mechanisms underlying their activation by various upstream signals and how they integrate various internal cues and external signals to control leaf senescence remain largely unknown.

In this study, we investigated the regulatory mechanisms by which light affects leaf senescence in Arabidopsis, focusing on the physiological and molecular roles of different light signal transduction components involved in regulating leaf senescence under high or low R:FR conditions. We found that the disruption of *FHY3* resulted in early leaf senescence under both high and low R:FR light conditions, indicating that FHY3 is a key negative regulator of leaf senescence. FHY3 inhibits leaf senescence by repressing the transcription of *WRKY28*, encoding a positive regulator of leaf senescence. This study provides important insights into the regulatory mechanisms by which plants integrate internal cues and environmental light signals to initiate and control leaf senescence through the FHY3–*WRKY28* transcriptional module.

RESULTS

FHY3 Negatively Regulates Age-Dependent Leaf Senescence

To investigate the regulatory mechanism by which light affects leaf senescence, we observed the leaf senescence phenotypes of various light signaling pathway-related Arabidopsis mutants grown under long-day (LD) conditions. The *fhy3-4* mutant exhibited early senescence compared to wild-type Nossen-0 (No-0) plants at various developmental stages (3, 5, and 7 weeks old, Supplemental Figure 1A). We also observed early leaf senescence in other mutants carrying *fhy3* null alleles in the No-0 (*fhy3-4, fhy3-6*, and *fhy3-10*) or Col-0 (*fhy3-1* and *fhy3-11*) ecotype backgrounds. In 5-week-old mature plants, the rosette leaves of all the *fhy3* mutants displayed obvious yellowing, while the rosette leaves of the wild-type control plants remained green (Figure 1A). Consistent with the early leaf senescence phenotype, the chlorophyll contents and maximum PSII efficiencies (indicated by the Fv/Fm ratio) of these *fhy3* mutants were also significantly lower than those of the wild-type control plants (Figures 1B and 1C).

To further explore whether the early leaf senescence of the *fhy3* mutants is caused by the functional disruption of FHY3, we observed the phenotypes of *FHY3pro:FHY3-YFP fhy3-4* (*FHY3-YFP*) transgenic plants. As expected, introducing the *FHY3-YFP* transgene into the *fhy3-4* mutant rescued its early leaf senescence phenotype to a large extent (Figures 1A and 1D; Supplemental Figure 1A). During leaf senescence, the transcript levels of well-studied marker genes *SAG13* and *PR1* were significantly higher in the *fhy3* mutants, while the transcript levels of a photosynthesis-related gene (*LIGHT-HARVESTING COMPLEX PHOTOSYSTEM II*, abbreviated as *LHCB4;1*) were significantly lower compared to wild-type No-0 plants (from leaf 3 to leaf 9) and the *FHY3-YFP* complementation line (from leaf 4 to leaf 7; Figure 1E; Supplemental Figure 1B). We also investigated the leaf senescence phenotypes of *FHY3pro:FHY3-GR fhy3-4* (*FHY3-GR*; glucocorticoid receptor) plants, a dexamethasone (DEX)-inducible complementation *fhy3-4* mutant line, after treatment with DEX or the mock control. After DEX treatment, the FHY3-GR fusion protein translocated from the cytosol to the nucleus and largely suppressed the early leaf senescence of *fhy3-4* (Supplemental Figure 1C). Together, these results suggest that FHY3 negatively regulates age-dependent leaf senescence.

FHY3 Negatively Regulates Light- and SA-Mediated Leaf Senescence

To investigate the physiological roles of FHY3 and its closest homolog FAR1 in light-mediated leaf senescence, we treated 3-week-old No-0, *fhy3-4, far1-2, fhy3-4 far1-2*, and *FHY3-YFP* plants grown under LD (with a R:FR ratio of ~2.5) conditions with high R:FR (~8.6) or low R:FR (~0.4) light for 2 weeks. No-0 plants senesced earlier when treated with low R:FR vs. high R:FR light, as

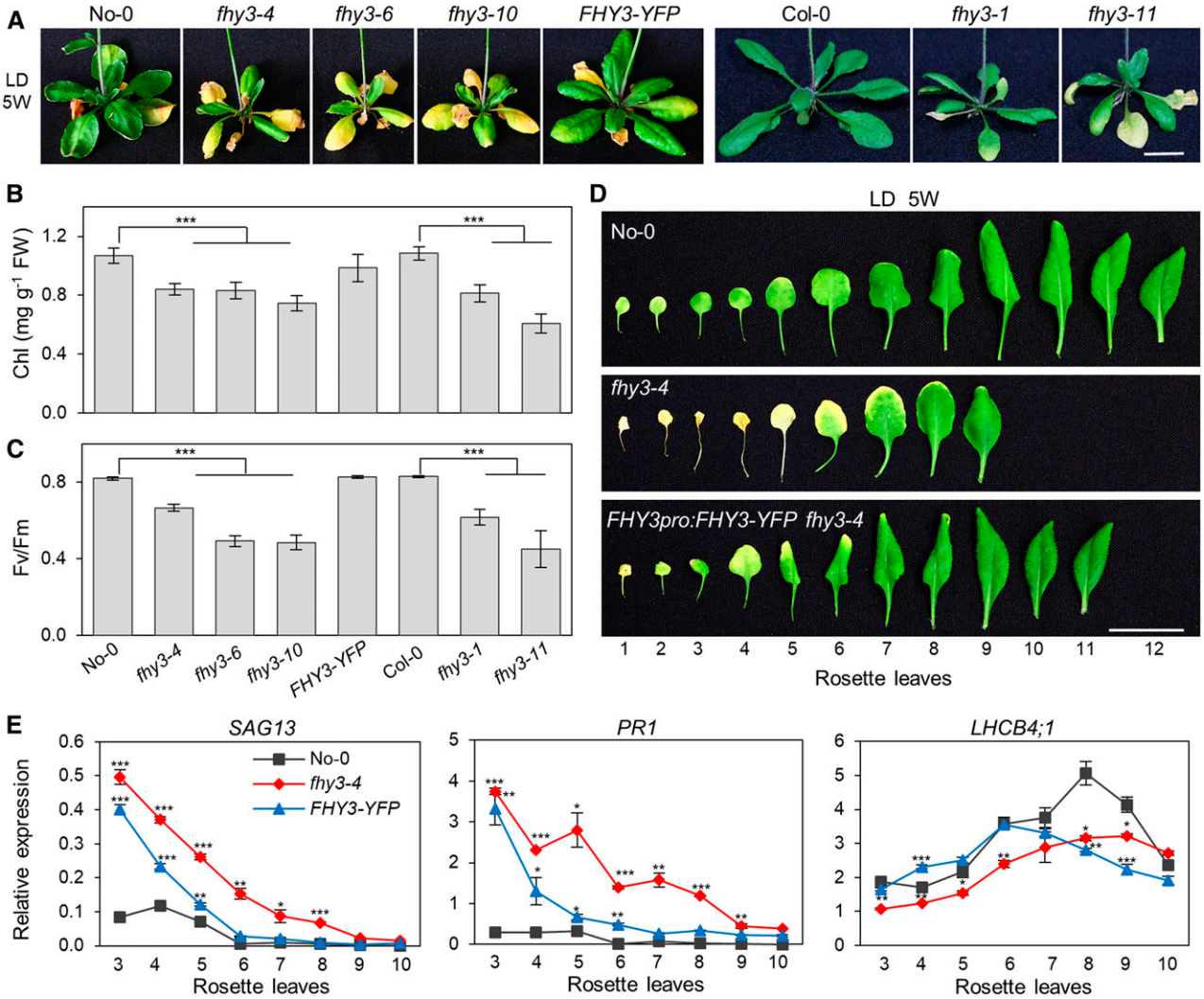

Figure 1. Disruption of *FHY3* Leads to Early Leaf Senescence in Arabidopsis.

(A) Leaf senescence phenotypes of 5-week-old (5W) *fhy3* plants grown under LD conditions. The *fhy3-4*, *fhy3-6*, and *fhy3-10* alleles are in the No-0 background, and the *fhy3-1* and *fhy3-11* alleles are in the Col-0 background. *FHY3-YFP* is a complementation line expressing *FHY3pro:FHY3-YFP* in the *fhy3-4* background. Bar = 2 cm.

(B) and **(C)** Whole-plant chlorophyll (Chl) contents **(B)** and Fv/Fm in the 5th leaf **(C)** of the various plants shown in **(A)**. Data are represented as means ±SD. ***$P < 0.001$.

(D) The morphology of different rosette leaves in No-0, *fhy3-4*, and *FHY3-YFP*. Bar = 2 cm.

(E) RT-qPCR analysis of the expression levels of *SAG13*, *PR1*, and *LHCB4;1* in the 3rd to 10th rosette leaves of various plants. Data are represented as means ±SD. The experiments were repeated at least three times with similar results, and representative results are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

evidenced by withered, yellow leaves and reduced chlorophyll contents (Figures 2A and 2B). These results suggest that a higher ratio of R:FR light inhibits senescence and a lower ratio of R:FR light induces senescence.

When compared to No-0 plants, the *fhy3-4*, *far1-2*, and *fhy3-4 far1-2* mutants (especially *fhy3-4*) senesced earlier under both high and low R:FR light conditions, as confirmed by measuring chlorophyll contents in the 7–8th rosette leaves (Figures 2A to 2C), indicating that FHY3 and FAR1 play important roles in negatively regulating leaf senescence in high or low R:FR light. Interestingly, compared to the wild type, the *far1-2* and *fhy3-4 far1-2* mutants underwent senescence earlier and had significantly reduced chlorophyll contents under high R:FR light vs. low R:FR light conditions (Figures 2A to 2C). Also, in *FHY3-YFP* complementation plants, the precocious leaf senescence and reduced chlorophyll contents of *fhy3-4* was rescued to a greater extent when treated with high R:FR light compared to low R:FR light conditions (Figures 2A to 2C). These results suggest that FHY3 and FAR1 play important roles in light-regulated leaf senescence, especially in inhibiting senescence mediated by high R:FR light.

We treated detached leaves of No-0, *fhy3-4*, *far1-2*, and *fhy3-4 far1-2* plants with white light or extended darkness for 5 d. After the

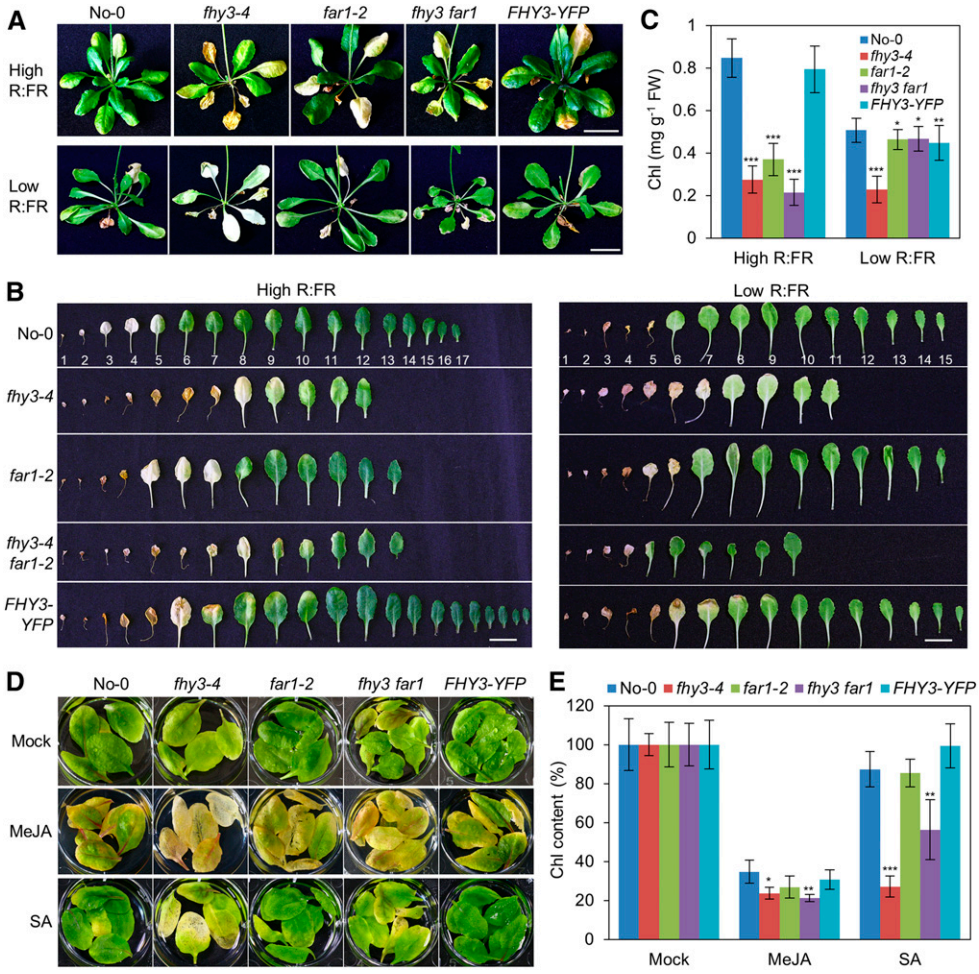

Figure 2. FHY3 Negatively Regulates Light- and SA-Mediated Leaf Senescence.

(A) and **(B)** Leaf senescence phenotypes of whole plants **(A)** and different rosette leaves **(B)** of various plants grown under high or low R:FR light conditions. Three-week-old plants grown under LD conditions were transferred to high or low R:FR LD conditions for 2 weeks and photographed. Bar = 2 cm.

(C) Total chlorophyll (Chl) contents in the 7th and 8th leaves of the various plants shown in **(A)**. Data are represented as means ± SD, $n \geq 7$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(D) and **(E)** The detached 7th and 8th leaves from 4-week-old plants grown under LD conditions were treated with 50 μM MeJA or 200 μM SA for 5 d, compared with mock treatment and used to analyze leaf senescence **(D)** and measure chlorophyll contents **(E)**. For **(E)**, data are represented as means ± SD, $n \geq 7$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

extended darkness treatment, the *fhy3-4* and *fhy3-4 far1-2* mutants showed considerably earlier precocious leaf senescence than No-0 plants. Consistent with this finding, the chlorophyll contents and photochemical efficiencies of the detached *fhy3-4* and *fhy3-4 far1-2* leaves were significantly reduced compared to wild-type No-0 leaves after extended darkness treatment (Supplemental Figure 2). These results indicate that FHY3 negatively regulates precocious senescence triggered by low R:FR light or extended darkness.

To investigate the physiological function of FHY3 in phytohormone-regulated leaf senescence, we treated detached leaves of No-0, *fhy3-4*, *far1-2*, *fhy3-4 far1-2*, and *FHY3-YFP* plants with SA and methyl jasmonate (MeJA). As shown in Figures 2D and 2E, the detached leaves of *fhy3-4* and *fhy3-4 far1-2* plants exhibited an earlier precocious senescence when treated with MeJA and SA than leaves treated with water (mock) or the phytohormone-treated leaves of No-0 and *FHY3-YFP* plants, as confirmed by measuring the chlorophyll contents of leaves (Figures 2D and 2E), indicating that FHY3 participates in JA- and SA-induced precocious senescence. These results suggest that FHY3 not only mediates age-dependent leaf senescence, but it is also involved in light- and SA-regulated leaf senescence.

WRKY28 Is a Direct Target of FHY3-Mediated Leaf Senescence

To investigate how FHY3 regulates leaf senescence at the molecular level, we searched the list of previously reported differentially expressed genes (DEGs) in the rosette leaves of 3-week-old *fhy3 far1* vs. No-0 plants (Wang et al., 2016) and DEGs during leaf

senescence (Breeze et al., 2011) and identified 2240 senescence-related genes that are also regulated (directly or indirectly) by FHY3 and FAR1. To narrow down the list of potential targets of FHY3 involved in regulating senescence, we compared these 2240 genes with previously reported ChIP-seq data for FHY3 in seedlings (Ouyang et al., 2011) and floral organs (Li et al., 2016). We identified 95 putative direct targets of FHY3 that might regulate leaf senescence, including *WRKY28* and *CYTOKININ RESPONSE FACTOR6* (*CRF6*; Supplemental Figure 3A; Supplemental Table 1).

We noticed that multiple, well-studied senescence related *WRKY* and *NAC* family genes are highly expressed in the rosette leaves of 3-week-old *fhy3 far1* plants (Supplemental Figure 3B). We verified their expression levels in 2-, 3-, and 5-week-old *fhy3* and *fhy3 far1* plants by RT-qPCR. As shown in Supplemental Figures 3C and 3D, only *WRKY28* was obviously upregulated in *fhy3* plants at all stages examined, which is consistent with the notion that it is a direct target of FHY3. The highly expression levels of other multiple senescence-related *WRKY* family genes (such as *WRKY53* and *WRKY75*) in *fhy3* and *fhy3 far1* plants were likely due to early precocious senescence, since they are not direct targets of FHY3. Since WRKY28 promotes SA biosynthesis by activating the expression of *SID2/ICS1* (van Verk et al., 2011), we hypothesized that *WRKY28* is a direct target of FHY3 that regulates leaf senescence.

Analysis of the promoter sequence of *WRKY28* revealed two typical FHY3 and FAR1 binding site (FBS; CACGCGC) *cis*-elements at –1034 nucleotides (FBS1) and –1015 nucleotides (FBS2) upstream of the ATG start codon (Figure 3A). Yeast one-hybrid assays showed that FHY3 and FAR1 specifically bind to these two FBS *cis*-elements. The mutation of FBS1 or FBS2 abolished or reduced the binding of FHY3 and FAR1 to the promoter region of *WRKY28*, indicating that these two FBS *cis*-elements (mainly FBS1) are critical for the specific binding of FHY3 and FAR1 to the *WRKY28* promoter in vitro (Figures 3B and 3C). To investigate whether FHY3 binds to the promoter region of *WRKY28* in vivo, we performed ChIP assays using the rosette leaves of *35Spro:3FLAG-FHY3-3HA* plants. ChIP-qPCR assays verified that the fragment containing the FBS *cis*-elements of the promoter of *WRKY28*, but not its exon region, was significantly enriched in the ChIP product of FHY3 (Figure 3D). This finding is consistent with previous ChIP-seq results for FHY3 in the seedlings (under FR light) and the floral organs (under white light) of *35Spro:3FLAG-FHY3-3HA* plants (Figure 3E; Ouyang et al., 2011; Li et al., 2016), indicating that *WRKY28* is a direct downstream target of FHY3.

To further explore the effect of FHY3 on *WRKY28* transcript levels, we performed transient expression assays in Arabidopsis protoplasts. As shown in Figure 3F, both FHY3 and FAR1 repressed the expression of *WRKY28pro:LUC*. To further explore the effect of FHY3 on *WRKY28* expression in rosette leaves during the aging process, we performed RT-qPCR using the 3rd to 10th leaves of No-0, *fhy3-4*, and *FHY3-YFP* complementation plants. The transcript levels of *WRKY28* were consistently higher in *fhy3-4* plants than in No-0 and *FHY3-YFP* plants (Figure 3G; Supplemental Figure 3C and 3D), indicating that FHY3 directly inhibits the expression of *WRKY28*.

WRKY28 Positively Regulates Leaf Senescence

To explore the physiological and molecular role of WRKY28 in the regulation of leaf senescence, we generated *WRKY28* null alleles in the No-0 background using a CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats [CRISPR]/associated protein 9) genome editing system controlled by an egg cell-specific promoter (Wang et al., 2015). We designed two single guide RNA (sgRNA) target sites in the first exon of *WRKY28*. Of the 52 T1 generation *WRKY28-CRISPR* plants, 11 independent lines exhibited delayed leaf senescence compared to the wild-type No-0 plants. DNA sequencing revealed a 31-bp deletion in the first exon of the *wrky28-c1* allele and a 7-bp deletion in the first exon of the *wrky28-c2* allele (Figure 4A; Supplemental Figures 4A and 4B). Under LD conditions, *wrky28-c1* and *wrky28-c2* plants displayed delayed leaf senescence compared to the wild-type No-0 plants (Figure 4B). Consistent with this finding, the chlorophyll contents of specific leaves were significantly higher in the *wrky28* null-allele plants versus wild-type plants (Figure 4C), while the transcript levels of senescence marker genes *SAG12*, *SAG13* and *SAG113* and WRKY28 direct target *SID2/ICS1* were much lower in the *wrky28* mutants than in wild-type No-0 leaves (Figure 4D).

To verify the positive role of WRKY28 in regulating leaf senescence, we constitutively expressed *WRKY28* under the control of the *35S* promoter in wild-type No-0 plants (*35Spro:WRKY28-3FLAG*, named *WRKY28-ox*). Under LD conditions, at least four individual *WRKY28-ox* lines (*ox1*, *ox6*, *ox7*, and *ox16*) exhibited earlier leaf senescence than wild-type No-0 plants (Figure 4E; Supplemental Figure 5). Consistent with the higher expression level of *WRKY28* and their precocious leaf senescence, the *WRKY28-ox* (*ox1* and *ox16*) plants had much lower chlorophyll contents and maximum PSII efficiencies (Fv/Fm) than the wild type and higher expression levels of senescence marker genes *SAG13*, *WRKY53*, and *WRKY75* (Figures 4F and 4G). In addition, we observed slightly delayed flowering in the *wrky28* null-allele mutants, but not in *WRKY28-ox* plants (Supplemental Figure 6). These results suggest that *WRKY28* positively regulates leaf senescence.

Disruption of *WRKY28* Rescues the Early Leaf Senescence of the *fhy3-4* Mutant

To explore whether FHY3 inhibits leaf senescence by repressing the transcription of *WRKY28*, we generated *wrky28* null alleles in the *fhy3-4* mutant background using CRISPR-Cas9 (Wang et al., 2015). After DNA sequencing, two *wrky28* null-alleles, *wrky28-c3* (with a 294-bp insertion in the first exon of *WRKY28*) and *wrky28-c4* (with a 133-bp deletion in the first exon of *WRKY28*), were identified in the *fhy3-4* background (Supplemental Figures 7A to 7D). Both *fhy3 wrky28-c3* and *fhy3 wrky28-c4* plants senesced much later than *fhy3-4* plants, with a similar phenotype to that of No-0 plants (Figure 5A; Supplemental Figure 7E). Consistent with the delayed leaf senescence phenotype, *SAG12* was down-regulated, while *LHCB4;1* was upregulated, in *fhy3 wrky28* vs. *fhy3-4* plants (Figure 5B). In addition, the SA biosynthesis gene *SID2/ICS1* (a direct target of WRKY28) and the well-studied SA response marker gene *PR1* were expressed at relatively high levels in *fhy3-4* plants but at significantly reduced levels in *fhy3*

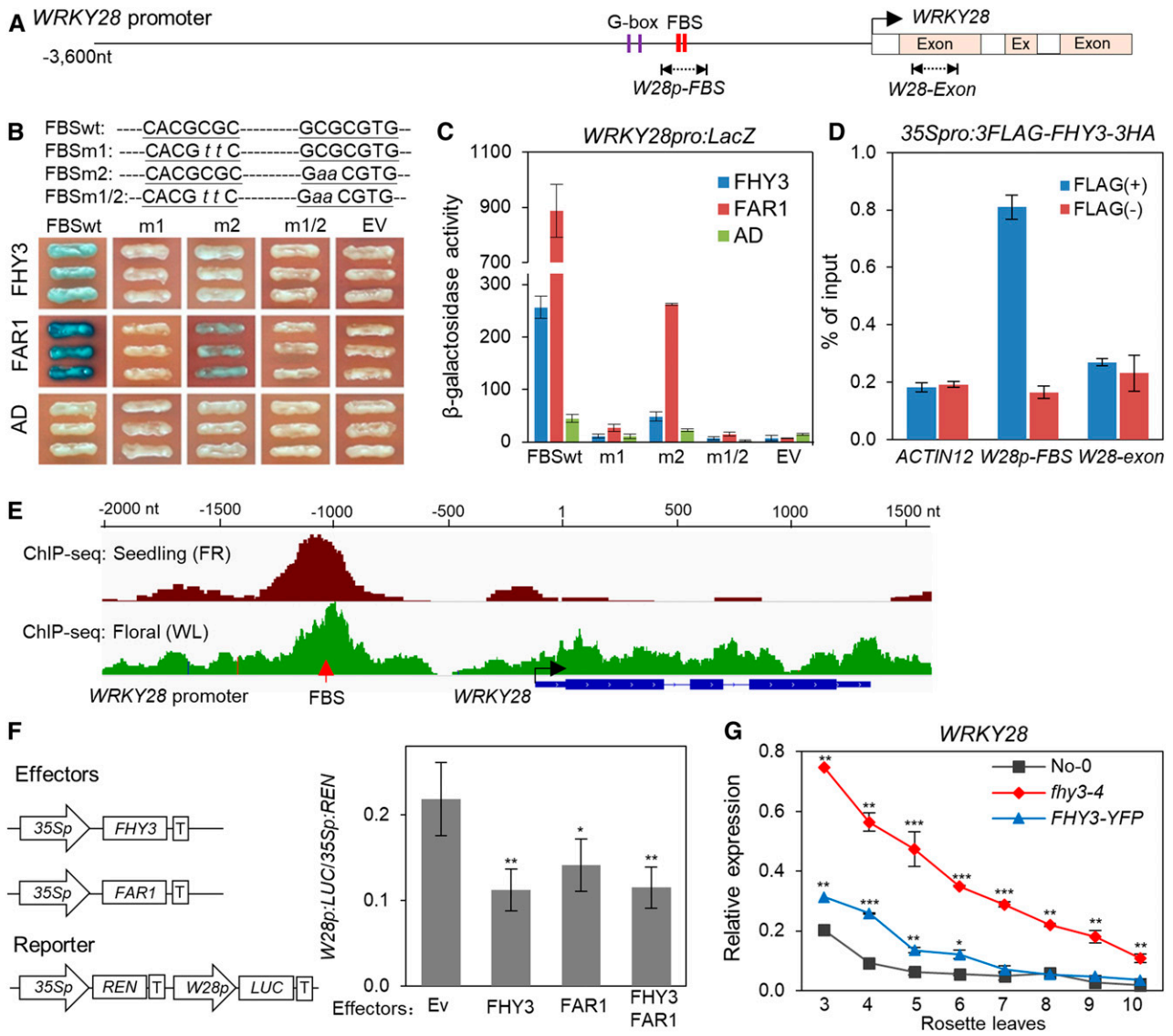

Figure 3. FHY3 Directly Binds to the *WRKY28* Promoter to Repress its Transcription.

(A) Diagram of the *WRKY28* promoter region showing the positions of FBS (red boxes), G-box motifs (purple boxes), and two ChIP amplicons (*W28p-FBS* and *W28-exon*; black dotted lines).

(B) Yeast one-hybrid assays showing that both FHY3 and FAR1 bind to the promoter region of *WRKY28* through two FBS *cis*-elements. The mutated nucleotide sequences of FBS *cis*-elements are shown in the top panel. AD, activation domain; EV, empty vector.

(C) Measurement of β-galactosidase activity shown in **(B)**. Data are represented as means ± SD, $n = 5$.

(D) ChIP-qPCR showing that FHY3 directly binds to the promoter region of *WRKY28* (*W28p-FBS*). *ACTIN12* and the exon region of *WRKY28* (*W28-exon*) were used as negative controls. Data are represented as means ± SD, $n = 3$.

(E) ChIP-seq showing that the promoter region of *WRKY28* is specifically enriched in the DNA immunoprecipitated by FHY3. The 4-d-old seedlings and the floral organs of *35Sp:3FLAG-FHY3-3HA fhy3* were used to perform the ChIP-seq in the previous studies (Ouyang et al., 2011; Li et al., 2016).

(F) Transient expression assay in Arabidopsis protoplasts showing that FHY3 and FAR1 repress the transcription of *WRKY28*. Data are represented as means ± SD, $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$.

(G) Relative expression level of *WRKY28* in different rosette leaves (from leaf 3 to leaf 10) of 4-week-old plants. Data are represented as means ± SD. The experiments were repeated at least three times with similar results, and representative results are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

wrky28 plants (Figure 5B). These results indicate that FHY3 inhibits senescence by repressing *WRKY28*, which might subsequently promote leaf senescence by promoting SA biosynthesis and the SA response.

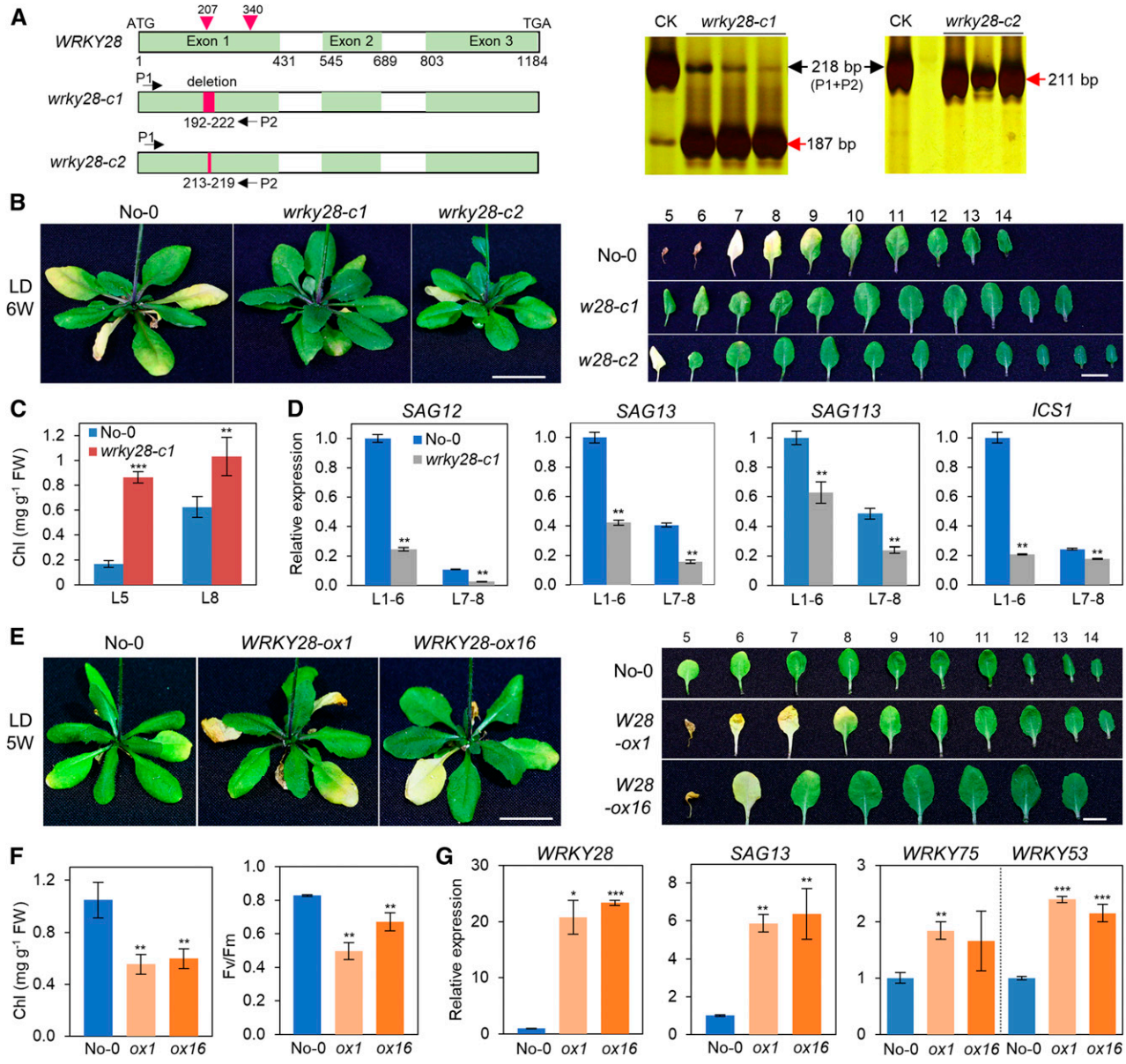

Figure 4. WRKY28 Positively Regulates Leaf Senescence.

(A) Identification of *wrky28-c1* and *wrky28-c2* null alleles generated via CRISPR-Cas9 in the No-0 background using PCR (right panels). The positions of the sgRNA (red triangle), deletion (red box), and primers (P1–P2) in the genomic region of *WRKY28* are shown in the left panel.

(B) Leaf senescence phenotypes of 6-week (6W)-old whole plants (left) and different rosette leaves (right) of various plants grown under LD conditions. Bar = 2 cm.

(C) Chlorophyll (Chl) contents of the 5th and 8th leaves of 6-week-old No-0 and *wrky28-c1* plants. Data are means ± SD, $n \geq 7$. $^{**}P < 0.01$, $^{***}P < 0.001$.

(D) Relative expression level of *SAG12, SAG13, SAG113*, and *ICS1* in various rosette leaves (L1–6 and L7–8) of 5-week (5W)-old No-0 and *wrky28-c1* plants. $^{**}P < 0.01$.

(E) Overexpression of *WRKY28* caused early leaf senescence. The leaf senescence phenotypes of 5-week-old *WRKY28-ox* transgenic plants (*ox1* and *ox16*) are shown. Bar = 2 cm.

(F) Total chlorophyll contents (left) and Fv/Fm (right) of the 6th and 7th leaves of No-0 and *WRKY28-ox* plants. Data are represented as means ± SD; $n \geq 7$ for the chlorophyll measurements and $n \geq 20$ for the Fv/Fm measurements. $^{**}P < 0.01$.

(G) Relative expression of *WRKY28, SAG13, WRKY75,* and *WRKY53* in 7-d-old No-0 and *WRKY28-ox* seedlings. Data are represented as means ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

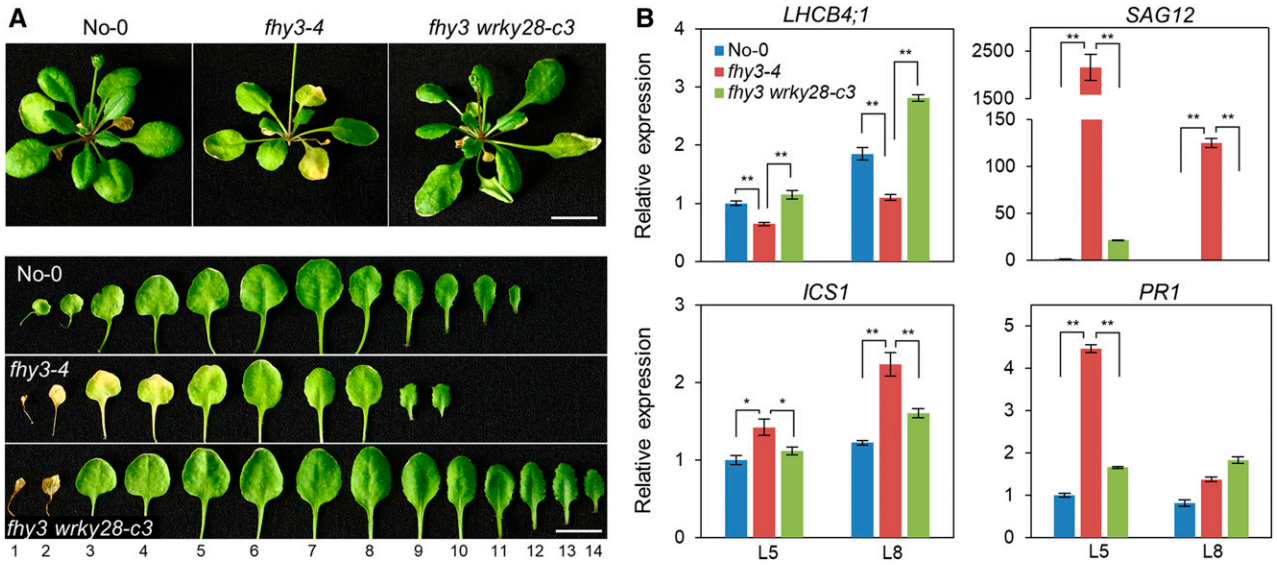

Figure 5. Disruption of *WRKY28* Rescues the Early Senescence Phenotype of *fhy3*.

(A) Senescence phenotypes of whole plants (top panel) and various rosette leaves (bottom panel) of 4-week-old plants grown under LD conditions. The mutation of *WRKY28* in *fhy3-4* plants was generated using CRISPR-Cas9. Bar = 2 cm.
(B) Relative expression levels of *LHCB4;1, SAG12, ICS1*, and *PR1* in the plants (5th and 8th leaves) shown in **(A)**. Data are represented as means ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

FHY3 and WRKY28 Control SA Biosynthesis and Accumulation, thus Regulating Senescence

Since WRKY28 positively regulates SA biosynthesis and leaf senescence, we investigated whether FHY3 inhibits leaf senescence by controlling SA biosynthesis or accumulation. Given that the SA contents and leaf senescence rates are significantly reduced in *sid2* and *S3H-ox* plants (Wildermuth et al., 2001; Zhang et al., 2013), we generated the *fhy3-4 sid2* double mutant and *fhy3-4 S3H-ox* lines to investigate the effect of SA on the FHY3-mediated inhibition of leaf senescence. As expected, the early senescence and decreased chlorophyll content of *fhy3-4* plants were largely rescued by the *sid2* allele or *S3H* overexpression (Figures 6A, 6B, 6D, and 6E). Consistent with this finding, the well-characterized senescence marker genes *SAG12* and *SAG13* were upregulated in *fhy3-4* but downregulated in *fhy3-4 S3H-ox* plants, while *LHCB4;1* was markedly downregulated in *fhy3-4* compared to No-0 and *fhy3-4 S3H-ox* plants (Figure 6C). The transcript levels of *SAG13* and *SENESCENCE-RELATED GENE2* (*SRG2*) were significantly higher in the rosette leaves of *fhy3-4* vs. No-0, while these levels were significantly lower in the *fhy3-4 sid2* double mutant (Figure 6F). These results indicate that FHY3 is involved in SA-mediated leaf senescence by regulating SA biosynthesis and accumulation.

Given that the *fhy3-4* mutant is sensitive to SA during leaf senescence (Figure 2D) and that *SID2/ICS1* is a direct target of WRKY28, we explored the role of WRKY28 in SA-induced leaf senescence. Overexpression of *WRKY28* led to a higher level of SA-induced leaf senescence compared to No-0 leaves, and the *WRKY28* mutation largely rescued the precocious leaf senescence of *fhy3-4* plants treated with SA (Supplemental Figure 8A), indicating that *WRKY28* plays an important role in inhibiting SA-induced senescence mediated by FHY3. The chlorophyll content measurements confirmed these results (Supplemental Figure 8B). These results suggest that FHY3 represses *WRKY28* expression to control SA biosynthesis and responses, subsequently repressing leaf senescence.

The FHY3–*WRKY28* Transcriptional Cascade Functions in Light-Mediated Leaf Senescence

Our results show that FHY3 and FAR1 mediate the inhibition of leaf senescence by high R:FR light and repress the induction of senescence under low R:FR light (Figures 2A to 2C). We then explored whether WRKY28 plays a similar role in the light-mediated regulation of leaf senescence. Three-week-old No-0, *fhy3-4*, *fhy3 wrky28-c3*, *wrky28-c1*, and *WRKY28-ox* plants grown under LD (a R:FR ratio of ~2.5) conditions were transferred to low or high R:FR light LD conditions and grown for 3 more weeks. Under low R:FR (a R:FR ratio of ~0.4) light conditions, all genotypes exhibited elongated petioles and reduced leaf blade size compared to plants grown under high R:FR light (Figure 7A). Under high R:FR (a R:FR ratio of ~8.6) light conditions, *fhy3-4* and *WRKY28-ox* plants exhibited accelerated leaf senescence, while the *wrky28* mutants displayed delayed leaf senescence compared to No-0 and *fhy3 wrky28-c3* plants (Figure 7B, left). The senescence phenotypes of plants grown under low R:FR light conditions showed a similar but much less dramatic trend compared to plants grown under high R:FR light conditions, except for *fhy3-4* and *WRKY28-ox* plants, which displayed strong senescence compared to No-0 under both high R:FR and low R:FR light conditions (Figure 7B). The senescence phenotypes were confirmed by measuring

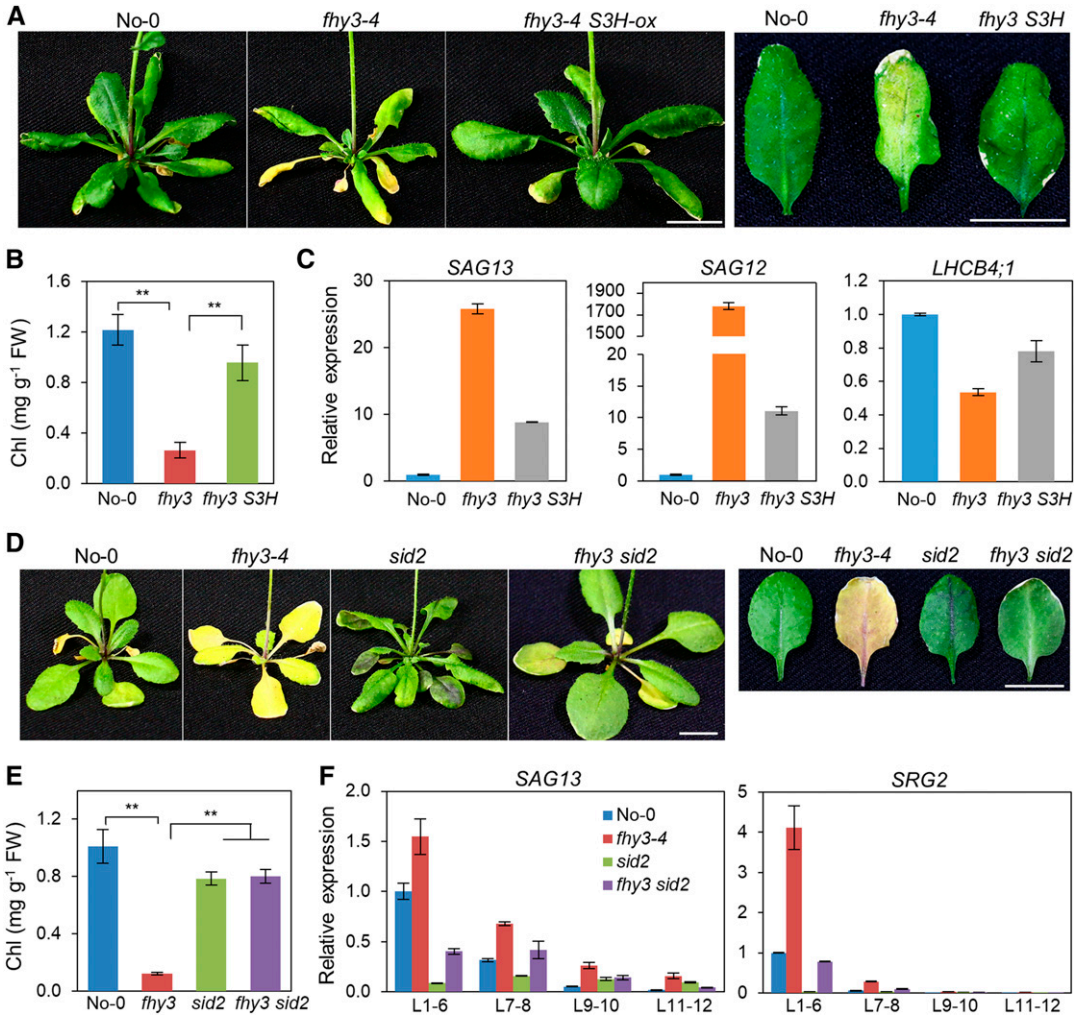

Figure 6. FHY3 Represses Leaf Senescence by Regulating SA Accumulation.

(A) to **(C)** *S3H* overexpression largely rescues the early senescence of *fhy3*. The senescence phenotypes of 5-week-old whole plants (left) and representative rosette leaves (L8, right) are shown in **(A)**. The contents of chlorophyll (Chl) and the relative expression levels of *SAG12, SAG13*, and *LHCB4;1* in the 8th leaf of various plants are shown in **(B)** and **(C)**, respectively. Data are represented as means ±SD. $^{**}P < 0.01$. Bar = 2 cm.

(D) to **(F)** Reducing SA biosynthesis by disrupting the function of *SID2/ICS1* largely rescues the early leaf senescence of *fhy3* (5-week-old) plants. The measurement of total chlorophyll contents in whole plants and relative expression levels of *SAG13* and *SRG2* are shown in **(E)** and **(F)**, respectively. Data are represented as means ± SD. $^{**}P < 0.01$. Bar = 2 cm.

chlorophyll contents (Figure 7C). These results indicate that even through both FHY3 and WRKY28 were effective in mediating high R:FR or low R:FR light-regulated senescence, the FHY3–*WRKY28* transcriptional regulatory module might play a predominant role in preventing leaf senescence under high R:FR light conditions.

The FHY3–*WRKY28* Transcriptional Module Integrates Age and Light Signals

To investigate the molecular roles of FHY3 and WRKY28 in regulating leaf senescence, we examined their expression profiles and the expression of previously identified key leaf senescence regulator WRKY75 and SA biosynthesis gene *SID2/ICS1* in rosette leaves at different developmental stages (from leaf 3 to leaf 11). As shown in Figure 8A, both *SID2/ICS1* and *WRKY75* were expressed at low levels in young rosette leaves (L9 and L11) and at considerably higher levels in senescing rosette leaves (L3 and L5). Although *WRKY28* transcript levels were much lower than those of *SID2/ICS1* and *WRKY75*, *WRKY28* expression gradually increased throughout leaf development, which is similar to the expression pattern of *FHY3* (Figure 8A).

To further assess the regulation of *FHY3* at the post-transcriptional level, we observed LUC protein accumulation in a transgenic plant harboring the *LUC* reporter gene fused with the *FHY3* coding region driven by the *FHY3* native promoter in the *fhy3-4* mutant background (*FHY3pro:FHY3-LUC fhy3-4*). As shown in Figure 8B, compared to other leaves, higher bioluminescence intensity of FHY3-LUC was detected in aging leaves (L5 and L6)

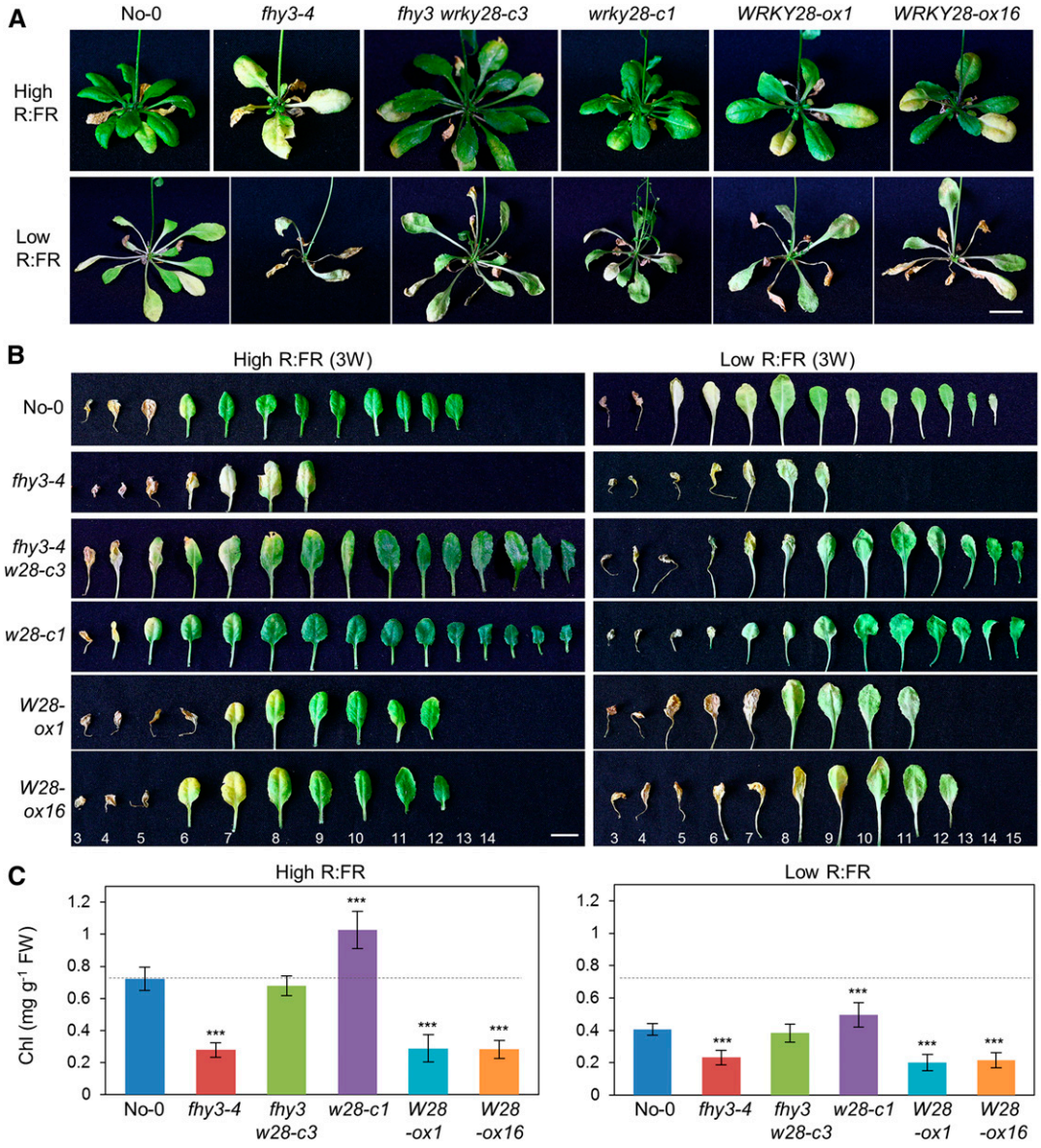

Figure 7. The FHY3–*WRKY28* Transcriptional Module Mediates Light-Regulated Leaf Senescence.

(A) and **(B)** the 3-week (3W)-old plants grown under LD conditions were transferred to high or low R:FR LD conditions and grown for 3 weeks. The senescence phenotypes of whole plants **(A)** and different rosette leaves **(B)** of various plants are shown. Bar = 2 cm.
(C) Chlorophyll (Chl) contents of the 7th and 8th leaves of various plants in **(B)**. Data are means ± SD, $n \geq 7$. ***$P < 0.001$.

and at the tips of mature leaves (L7 to L10), where senescence symptoms usually initiate. These results indicate that *FHY3* expression is induced by aging signals at both the transcriptional and posttranscriptional levels.

As FHY3 plays an important role in photomorphogenesis (Wang and Deng, 2002), we investigated whether the FHY3–*WRKY28* transcriptional module is present at the seedling stage. As shown in Figure 8C, RT-qPCR analysis revealed that *WRKY28* was upregulated in 7-d-old *fhy3-4* seedlings compared to the wild type. *SAG13* was also upregulated in *fhy3-4* seedlings, suggesting that FHY3- and WRKY28-regulated leaf senescence also affect the transcription of *SAG13*. However, the transcript levels of genes encoding other well-studied key senescence regulators, including *WRKY30*, *WRKY51*, *WRKY53*, *WRKY75*, *NAC016*, *NAC047*, and *EIN3*, were not significantly higher in *fhy3-4* seedlings compared to the wild type (Figure 8C). These results indicate that the high expression levels of these genes in *fhy3-4* at the adult stage (Supplemental Figure 3) are due to precocious leaf senescence.

As the FHY3–*WRKY28* transcriptional module is present at the seedling stage, we subjected 7-d-old *fhy3*, *far1*, *fhy3 far1*, and *WRKY28-ox* seedlings to continuous growth in high R:FR (a R:FR ratio of ~8.62) or low R:FR (a R:FR ratio of ~0.12) light conditions

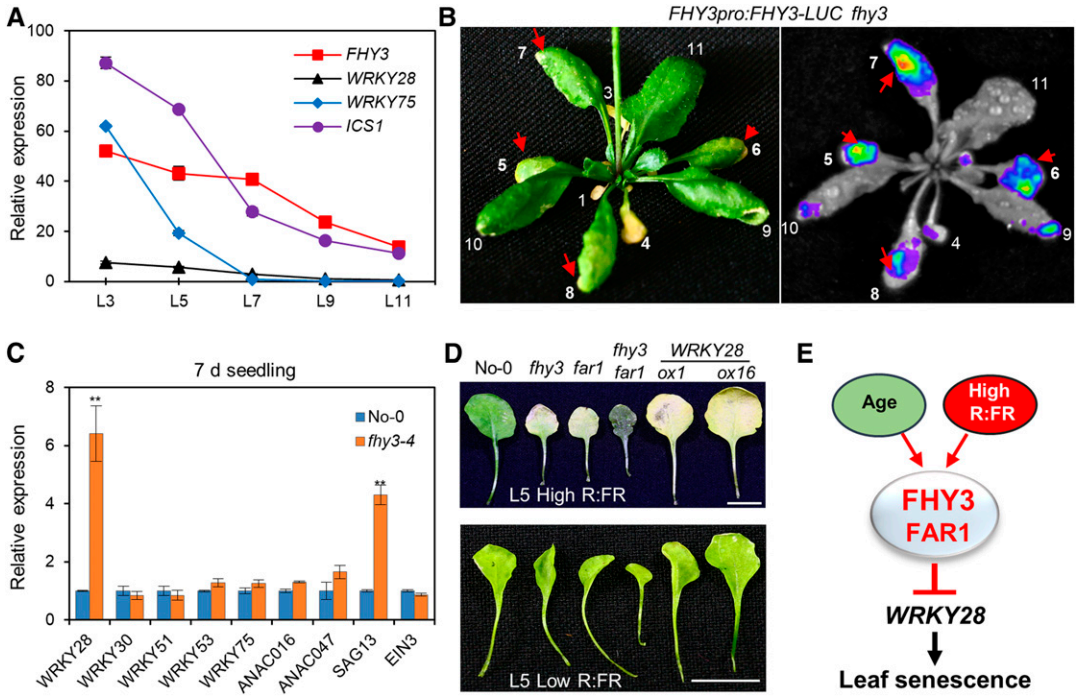

Figure 8. The FHY3–*WRKY28* Transcriptional Module Integrates Age and Light Signals to Repress Leaf Senescence.

(A) Relative expression levels of *FHY3, WRKY28*, *WRKY75*, and *ICS1* in different rosette leaves of 4-week-old wild-type No-0 plants. Leaf yellowing was observed in the tips of the 7th leaf, one fourth of the 5th leaf, and half of the 3rd leaf. The experiments were repeated at least three times with similar results and representative results are shown. Data are represented as means ± SD.

(B) Bioluminescence analysis showing the robust expression of FHY3-LUC (indicated by red arrows) in different rosette leaves of 5-week-old plants.

(C) Relative expression levels of senescence-related genes in 7-d-old *fhy3* seedlings. Data are represented as means ± SD. The experiments were repeated at least three times with similar results, and representative results are shown. $^{**}P < 0.01$.

(D) FHY3 and WRKY28 are required in high R:FR light inhibited leaf senescence. The 7-d-old seedlings of various plants were transferred to high R:FR (a R:FR ratio of ~8.62) or low R:FR (a R:FR ratio of ~0.12) light LD conditions and grown for 4 weeks; representative leaves (L5) are shown. Bar = 1 cm.

(E) A model showing how the FHY3–*WRKY28* transcriptional module integrates age and high R:FR light signals to repress leaf senescence.

for 4 weeks (Figure 8D; Supplemental Figure 9). As shown in Figure 8D, under high R:FR light conditions, representative rosette leaves (L5) of *fhy3*, *far1*, *fhy3 far1*, and *WRKY28-ox* plants exhibited early leaf senescence compared to those of the wild-type No-0 plants (top). Under very low R:FR light conditions (lower panel), the leaf senescence phenotype of these representative leaves (L5) was indistinguishable from that of No-0 plants, although the shape and size of these leaves differed from leaves grown under high R:FR light conditions. These results further support the notion that the FHY3-*WRKY28* transcriptional module plays a more important role in preventing leaf senescence under high R:FR vs. low R:FR light conditions.

DISCUSSION

Leaf senescence, the final stage of leaf development, is tightly controlled by the integration of numerous internal and external signals (Guo and Gan, 2005; Schippers et al., 2015; Woo et al., 2019). Although several key components involved in age- or stress-triggered leaf senescence have been identified (Kim et al., 2018; Sade et al., 2018; Woo et al., 2019), the key components mediating the inhibition of senescence in high R:FR light conditions have been unclear. In this study, we demonstrated that the light signaling protein FHY3 and its closest homolog FAR1 negatively regulate age- and light-mediated leaf senescence by repressing the transcription of *WRKY28*, which subsequently promotes SA biosynthesis and leaf senescence.

Arabidopsis FHY3 and FAR1 Are Negative Regulators of Leaf Senescence

In this study, we showed that the disruption of *FHY3* caused early leaf senescence in Arabidopsis under white light and high R:FR light conditions. Complementation of the *fhy3* mutant with the *FHY3-YFP* or *FHY3-GR* fusion constructs largely rescued its early leaf senescence phenotype (Figures 1 and 2; Supplemental Figure 1), indicating that FHY3 is a negative regulator of leaf senescence. Consistent with this notion, *FHY3* was expressed at higher levels in aging leaves or senescent sections of leaves versus other leaves at both the transcriptional and posttranscriptional levels (Figures 8A and 8B). FAR1, the closest homolog of FHY3, usually plays a redundant (but weaker) role with FHY3 in many cellular processes involved in plant growth and development (Wang and Wang, 2015; Ma and Li, 2018). Although *far1* plants

exhibited a subtle early leaf senescence phenotype under white-light conditions, they displayed a strong early senescence phenotype under high R:FR light conditions (Figure 2), indicating that FAR1 is also a negative regulator of leaf senescence. Consistently, the loss of function of FHY3 and FAR1 increased plant sensitivity to extended darkness, SA, and MeJA treatment, leading to more rapid precocious leaf senescence than wild-type No-0 plants (Figures 2D and 2E; Supplemental Figure 2A and 2B). These results indicate that FHY3 and FAR1 are involved in regulating leaf senescence.

Light is one of the most important environmental factors that determine when and how leaf senescence is initiated and regulated (Weaver and Amasino, 2001; Brouwer et al., 2012). After a 2- or 3-week-long high or low R:FR light treatment in adult stage plants, the *fhy3* mutants exhibited early leaf senescence under both high R:FR and low R:FR light treatment compared to wild-type No-0 plants (Figures 2A, 2B, 7A, and B7B), which further indicates that FHY3 acts as a repressor of light-regulated leaf senescence. The disruption of *FAR1* resulted in a more obvious early leaf senescence phenotype under high R:FR light vs. low R:FR light conditions compared to wild-type No-0 plants, indicating that both FHY3 and FAR1 are essential for light-regulated leaf senescence, primarily for preventing leaf senescence under high R:FR light conditions (Figures 2A and 2B). More importantly, at the seedling stage, when the process of leaf senescence has not been determined or initiated, after a 4-week high or low R:FR treatment, both the *fhy3* and *far1* mutants exhibited early leaf senescence under high R:FR light but not low R:FR light conditions, suggesting that FHY3 and FAR1 specifically prevent the initiation of leaf senescence under high R:FR light conditions (Figures 8D and 8E; Supplemental Figure 9).

Although we demonstrated that Arabidopsis FHY3 and FAR1 specifically prevent the initiation of leaf senescence under high R:FR light conditions, the regulatory mechanism by which they respond to high R:FR light and the direct molecular link between phytochromes and FHY3/FAR1 remain unknown. The disruption of phyB, the major photoreceptor mediating the continuous R light response, caused early senescence under R light enrichment or extended darkness (Brouwer et al., 2014; Sakuraba et al., 2014; Lim et al., 2018). phyB mediates darkness-induced senescence by affecting the degradation of its interacting proteins PIF4 and PIF5 (Sakuraba et al., 2014; Song et al., 2014; Zhang et al., 2015). In this study, we revealed that FHY3 plays an important role in high R:FR-inhibited leaf senescence, which is consistent with the physiological role of phyB in preventing senescence when plants are exposed to R light (Lim et al., 2018). Indeed, FHY3 act downstream of light-stable phytochromes, such as phyB, during circadian clock regulation (Allen et al., 2006; Siddiqui et al., 2016). In addition, recent studies demonstrated that FHY3 is involved in low R:FR-induced hypocotyl elongation by affecting the transcription of *PHYTOCHROME RAPIDLY REGULATED1* (*PAR1*) and *PAR2* (Liu et al., 2019; Ma et al., 2019). Therefore, it is likely that FHY3 genetically or physically interacts with phyB or participates in the phyB–PIF4 signaling pathway, thus regulating leaf senescence and shade avoidance responses.

In Arabidopsis, the photoreceptor phyA mediates the plant response to continuous FR light. phyA is thought to play a role in light-regulated leaf senescence, and *phyA* mutants exhibit weakly altered leaf senescence or a similar trend to that of wild-type control plants (Brouwer et al., 2014; Sakuraba et al., 2014; Lim et al., 2018). Given that FHY3 and FAR1 are important transcription factors in the phyA-mediated FR light signaling pathway (Wang and Deng, 2002; Lin et al., 2007), it would be useful to further explore the leaf senescence phenotypes of various plants such as *phyA fhy3*, *phyA FHY3-ox*, or *phyA-ox fhy3* under long-term high or low R:FR light conditions. In conclusion, further studies of the genetic and molecular relationships among FHY3, phyA, and phyB will be required to explore the regulatory mechanisms underlying how high R:FR light inhibits leaf senescence.

FHY3 Regulates Age- and Light-Mediated Leaf Senescence via WRKY28

In this study, we demonstrated that WRKY28, a WRKY transcription factor that functions downstream of FHY3, is a positive regulator of leaf senescence. The disruption of *WRKY28* in either wild-type No-0 or *fhy3* plants delayed leaf senescence, whereas the overexpression of *WRKY28* accelerated leaf senescence (Figure 4). In addition, the number of rosette leaves at bolting was slightly higher in *wrky28-c1*, *wrky28-c2*, and *fhy3 wrky28-c3* plants compared to wild-type No-0 plants (Figures 4B and 5A; Supplemental Figure 6), indicating that WRKY28 also promotes flowering under LD conditions. Interestingly, the number of rosette leaves at bolting was not significantly lower in *WRKY28-ox* plants compared to wild-type No-0 plants (Figure 4E; Supplemental Figure 7), suggesting that the accelerated leaf senescence of *WRKY28-ox* plants was not caused by altered flowering time. Therefore, WRKY28 positively regulates the process of leaf senescence, which is independent from its effect on flowering time regulation.

Arabidopsis WRKY28 is a type IIc WRKY transcription factor whose expression is highly induced by drought, oxalic acid, oxidative stress, and pathogens (Babitha et al., 2013; Chen et al., 2013; Timmermann et al., 2019). WRKY28 directly binds to W-box *cis*-elements located in the promoter of *SID2*/*ICS1* and activates its expression, subsequently promoting the SA biosynthesis and mediating various stress responses (van Verk et al., 2011; Babitha et al., 2013; Chen et al., 2013; Timmermann et al., 2019). Based on the leaf senescence phenotype and chlorophyll contents, *WRKY28-ox* plants had increased sensitivity to SA, whereas *fhy3 wrky28* plants had decreased sensitivity to SA compared to wild-type No-0 and *fhy3-4* plants, respectively (Supplemental Figure 8), which is consistent with the decreased content of SA in the other null-allele mutants of *WRKY28* (Zhao et al., 2018). Together, these results support the idea that WRKY28 positively regulates leaf senescence by promoting SA biosynthesis. SA contents were slightly higher in adult *fhy3* and *fhy3 far1* plants compared to wild-type No-0 plants (Wang et al., 2016). In addition, the decrease in SA content in the *fhy3 sid2* double mutant or the disruption of *WRKY28* in the *fhy3* mutant rescued the early senescence of *fhy3-4* mutants to a great extent (Figures 5 and 6). These genetic and physiological results support the idea that the FHY3–*WRKY28* transcriptional module regulates leaf senescence by affecting the transcription of *SID2*/*ICS1* and the biosynthesis of SA (Figure 8E). In addition, *WRKY28-ox* plants exhibited early senescence under high R:FR light conditions, which is very similar to the phenotypes

of the *fhy3* mutant (Figures 7 and 8D; Supplemental Figure 9). These results support the idea that *WRKY28* acts downstream of FHY3 to mediate age- and light-regulated leaf senescence (Figure 8E). In summary, our findings reveal that the light signaling protein FHY3 integrates internal age cues and environmental light signals to repress the transcription of *WRKY28* and SA biosynthesis, thus negatively regulating leaf senescence.

METHODS

Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) mutants *fhy3-4*, *far1-2*, *fhy3-4 far1-2*, *fhy3-6*, and *fhy3-10*, all in the No-0 background, and *fhy3-1* and *fhy3-11* in the Col-0 background were previously described by Wang and Deng, (2002), Lin et al. (2007), and Ma et al. (2017). The transgenic Arabidopsis lines *35Spro:3FLAG-FHY3-3HA fhy3-4*, *FHY3pro:FHY3-YFP fhy3-4*, *FHY3pro:FHY3-GR fhy3-4*, *FHY3pro:FHY3-LUC fhy3-4*, and *35Spro:S3H-ox fhy3 far1* were previously described by Lin et al. (2007), Li et al. (2011), and Ma et al. (2016). The Arabidopsis lines *fhy3-4 sid2* and *fhy3-4 S3H-ox* were generated through genetic crosses. For phenotypic analysis, the seeds were sown in soil after a 2-d incubation at 4°C and grown under LD (16-h light/8-h dark) conditions with 100 μmol/m^2/s cool-white fluorescent light at 22°C. For light quality treatments, the white light was supplemented with R light (660 nm) or FR light (730 nm) to a total light radiation of 100 μmol/m^2/s. The ratios of high, low, and very low R:FR were ~8.6, 0.4, and 0.1, respectively.

Construction of Plasmids and Generation of Transgenic Plants

To generate the transgenic Arabidopsis line *WRKY28-ox*, the full-length coding region of *WRKY28* (without the stop codon) was PCR amplified from No-0 plants and inserted into the *Kpn*I- and *Sal*I-digested pPZP211-3FLAG vector (Ma et al., 2016) to produce *pPZP211-35Spro:WRKY28-3FLAG*. The resulting construct was transferred into *Agrobacterium tumefaciens* GV3101 and used to transform No-0 plants by the floral dip method (Clough and Bent, 1998). More than 30 individual T1 generation plants were identified based on antibiotic resistance. The seedlings of the T2 generation were used to perform RT-qPCR and immunoblotting assays, leading to the identification of *WRKY28* overexpression lines. T3 generation transgenic lines were used for phenotypic analyses of leaf senescence.

To generate *wrky28* null alleles in the wild-type No-0 or *fhy3-4* background, an egg cell–specific promoter-controlled CRISPR-Cas9 system was used (Wang et al., 2015). Two guide RNA target sites in the first exon of *WRKY28* were selected to perform the gene editing (Supplemental Table 2). The resulting construct was transformed into No-0 or *fhy3-4* plants by the floral dip method. The *wrky28-c1*, *wrky28-c2*, *fhy3-4 wrky28-c3*, and *fhy3-4 wrky28-c4* mutants were identified by PCR, confirmed by DNA sequencing, and used for further analysis. All primers used for vector construction and mutant identification are listed in Supplemental Table 2.

Analysis of Leaf Senescence Phenotypes

To analyze leaf senescence after extended darkness, the 7th and 8th rosette leaves were detached from 4-week-old plants, placed in Petri dishes containing wet filter paper, and incubated in the dark or light for 5 d. For the hormone-induced leaf senescence assays, detached 7th and 8th rosette leaves were floated on 3 mL of 200 μM SA or 50 μM MeJA. The Petri dishes were sealed with Parafilm tape and incubated under cool-white light for 5 d at 22°C. For the DEX treatment assay, the rosette leaves of plants grown under LD conditions were sprayed with 30 μM DEX or DMSO (Mock) once a day for 8 d.

For chlorophyll measurements, the leaves were incubated in 95% (v/v) ethanol for 3 d in the dark. The absorbances were measured at 665 and 649 nm. The chlorophyll contents were calculated according to the following ratio: (6.63A665 + 18.08A649)/g fresh weight. The Fv/Fm values were determined using a LI-COR 6400 photosynthesis fluence system.

Yeast One-Hybrid Assays

Yeast one-hybrid assays were performed as previously described by Lin et al. (2007) and Ma et al. (2016). The AD-FHY3 and AD-FAR1 constructs were previously described by Ma et al. (2016). To generate various types of *WRKY28pro:LacZ* reporters, a set of ~60-bp fragments containing wild-type or mutated FBS *cis*-elements from the *WRKY28* promoter were synthesized and inserted into *Kpn*I-*Sal*I-digested pLacZi-2u.

Transient Expression Assay in Arabidopsis Protoplasts

To generate the *WRKY28pro:LUC* reporter, a fragment containing the *WRKY28* promoter (~1.2 kb) was amplified from genomic DNA from wild-type No-0 plants and inserted into *Kpn*I- and *Sal*I-digested pGreen-0800 (Hellens et al., 2005). To generate the effectors *35Spro:FHY3* or *35Spro:-FAR1*, the full-length coding regions of *FHY3* or *FAR1* were amplified from AD-FHY3 or AD-FAR1, respectively. These two fragments were inserted into *Kpn*I-*Sal*I- or *Kpn*I-*BamH*I-digested pPZP211 to produce *pPZP211-35Spro:FHY3* or *pPZP211-35Spro:FAR1*, respectively. Arabidopsis protoplasts were cotransformed with various combinations of effectors and reporters using the polyethylene glycol method (Cao et al., 2016). The ratio of LUC driven by the *WRKY28* promoter to *Renilla* LUC (REN) driven by the 35S promoter were measured to determine the transcriptional activities.

ChIP Assay

The ChIP assay was performed as previously described by Li et al. (2011). Approximately 2 g of rosette leaf tissue from 3-week-old *35Spro:3FLAG-FHY3-3HA* plants was collected at Zeitgeber time 4 (ZT4; ZT0 indicates the start of the light phase). Anti-FLAG monoclonal antibodies (Sigma-Aldrich) were used to perform an immunoprecipitation of the 3FLAG-FHY3-3HA fusion protein. The DNA products precipitated by FHY3 were used to perform qPCR assays. The enrichment of specific DNA fragments in the promoter or exon regions of *WRKY28* or *ACTIN12* were detected by qPCR. The ChIP-seq peak of FHY3 located in the promoter region of *WRKY28* was reanalyzed based on previous studies by Ouyang et al. (2011) and Li et al. (2016) and visualized using the Integrative Genomics Viewer program.

qPCR Assay

The RT-qPCR and qPCR assays were performed as previously described by Ma et al. (2016). *UBQ1* expression was used as an internal control. All primers used in the qPCR assays are listed in Supplemental Table 2.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or EMBL/GenBank data libraries under the following accession numbers: *FHY3* (AT3G22170), *FAR1* (AT4G15090), *WRKY28* (AT4G18170), *ICS1*/*SID2* (AT1G74710), *UBQ1* (AT3G52590), and *ACTIN12* (AT3G46520).

Supplemental Data

Supplemental Figure 1. FHY3 negatively regulates age-dependent leaf senescence.

Supplemental Figure 2. FHY3 negatively regulates extended darkness-induced leaf senescence.

Supplemental Figure 3. Expression profiling analysis of *SAGs* in *fhy3* and *fhy3 far1*.

Supplemental Figure 4. Identification of two *wrky28* mutants generated in No-0 via CRISPR-Cas9.

Supplemental Figure 5. Constitutive overexpression of *WRKY28* causes early leaf senescence.

Supplemental Figure 6. Disruption of *WRKY28* leads to delayed flowering.

Supplemental Figure 7. Identification of *wrky28* null alleles generated in *fhy3-4* using CRISPR-Cas9.

Supplemental Figure 8. Disruption of *WRKY28* rescued the SA-hypersensitivity-induced precocious leaf senescence of *fhy3-4* plants.

Supplemental Figure 9. The FHY3-*WRKY28* transcriptional module prevents leaf senescence under high R:FR light conditions.

Supplemental Table 1. Genes directly targeted and regulated by FHY3 during leaf senescence.

Supplemental Table 2. All primers used in this study.

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AUTHOR CONTRIBUTIONS

T.T. carried out most of the experiments, analyzed the data, and wrote the article; L.M. and Y.L. identified the transgenic plants; G.L. conceived the project and wrote the article.

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