Supporting Information Appendix

Circadian control of *ORE1* by PRR9 positively regulates leaf senescence in *Arabidopsis*

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SI Materials and Methods

Plant materials and growth condition

For constitutively expressing line, *CsV*:*PRR9-GFP* and *CsV:ORE1-HA* were constructed using LR recombination (GATEWAY; Invitrogen, Carlsbad, CA, USA), and introduced into the *prr9-1* and *ore1-2* plants by *Agrobacterium tumefaciens*-mediated transformation, respectively. For inducible transgenic line, full-length *PRR9-LUC*, a translational fusion of LUC to the C-terminus of PRR9, was subcloned into the ecdysone agonist-inducible vector, VGE/linker (1) and introduced into Col-0 plants by *Agrobacterium tumefaciens*-mediated transformation. The *prr9-1ORE1-ox*, and *prr9-1ore1-2* plants were generated by genetic cross and characterized using both physiological phenotypes and PCR-based genotyping. The *ore1-2* mutation was identified using primers; 5'-TGTGAGAGACCGGAAATACC-3' and 5'-GTCTTTTGTCTTCGGTTTCT-3'; in this reaction, the *ore1-2* mutation did not show the PCR band. The *prr9-1* mutant was isolated using PCR as described previously (2). *Arabidopsis thaliana* plants were grown in an environmentally controlled growth room at 22 °C with a 16 h light/8 h dark cycle (long day: LD) using 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent light. All physiological experiments were carried out using the third and fourth rosette leaves. Leaf samples were obtained by cutting leaves at approximately the middle of the petiole with a sharp scalpel to minimize wounding effects.

Assay of age-dependent senescence

The photochemical efficiency of photosystem II (PSII) was deduced from chlorophyll fluorescence (3) using an Imaging-PAM chlorophyll fluorometer (Heinz Walz GmbH). The ratio of the maximum variable fluorescence to the maximum yield of fluorescence was used as a measure of photochemical efficiency of PSII (4).

Analysis of gene expression

Total mRNA was extracted from the leaves using WelPrep (Welgene, Daegu, Korea). Contaminating DNA was removed by digestion with DNase I (Ambion, Austin, TX, USA). For each sample, 0.75 μg of total mRNA was reverse-transcribed using ImProm II reverse transcriptase (Promega, Madison, WI, USA). The quantity of each transcript in a sample was measured using real-time PCR with universal SYBR Supermix (Bio-rad, Hercules, CA, USA) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA). Fold changes in gene expression were calculated using the comparative CT method, with normalized against *ACT2* (At3g18780) expression.

Luminescence assay

Transgenic plants expressing luciferase under the control of the *ORE1* promoters were used in this assay. Luminescence detection from attached third and fourth leaves is almost impossible because newly emerged leaves cover those targeted leaves. Therefore, we performed the luminescence assays with detached leaves; oscillation patterns of core clock and output genes have been previously shown to be similar between transcript levels in attached leaves and luminescence in detached leaves (5). The third and fourth rosette leaves were excised at their petioles from transgenic plants and transferred to 24-well microplates containing 500 μM luciferin (Gold Biotechnology, St. Louis, MO, USA) under continuous conditions (22 °C, 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from white LED, D.K.I. international Co.). Luminescence images were acquired every hour for 10 min and luminescence intensities from each leaf were counted by MetaView system. To monitor changes in clock gene expression, the plants were then transferred to continuous white light at the same light intensity to measure rhythmic changes. Luminescence images were acquired every hour for 4 days and luminescence intensities from each leaf were imported into the Biological Rhythms Analysis Software System (BRASS) (6). Circadian period lengths were calculated using the FFT-NLLS suite (7).

Yeast one-hybrid assays

The DupLEX-A system (OriGene Technologies, USA) was used for Y1H assays and with slight modifications. For Y1H experiments, full-length cDNAs of core clock component genes were cloned into the pJG4-5 prey vector, which includes a B42 transcriptional activation domain from CCA1, LHY, PRR7, PRR9, TOC1 and GI in pCR-CCD vector (8) by LR recombination reaction. For bait, a 2 kb of the *ORE1* promoter (9) and 2 kb of the *MIR164B* promoter were cloned separately into the lacZ (*β-galactosidase*) reporter plasmid pSH18-34. The yeast strain EGY48 (*MATa*, *trp1*, *his3*, *ura3*, *leu2*::6 LexAop-*LEU2*) was transformed with the indicated combinations of plasmids. Interactions were tested on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (*X-gal*) medium (10).

Transient expression assay in *Arabidopsis* protoplasts

For luciferase reporter constructs, the promoter of *ORE1* were amplified from genomic DNA, cloned into *pCR-CCD F* (11), and recombined into the gateway version of the *pGreen0800::LUC* vector (9, 12), which contains *35Sp::RLuc* (Renilla *luciferase*) as an internal control. *Arabidopsis* protoplasts were isolated and transfected as described (13). Transfected protoplasts with the *ORE1p:LUC* reporter and an effector plasmid expressing CCA1, GI, or PRR9 or vector control were transferred to 96-well

microplates containing 50 μM luciferin (Gold Biotechnology) or 5 μM coelenterazine native (NanoLight Technology). Luminescence images were acquired in continuous light and luminescence intensities were counted by MetaView system. To make mutagenized G box (mG box) *ORE1* promoter, we changed G box sequence (CACGTG) to TCGAGC by using a QuickChange® site-directed mutagenesis kit (Stratagene, http://www.stratagene.com/) according to the manufacturer's instructions. For transactivation assay of *ORE1* promoter, transfected protoplasts were incubated for 6 h at 22 ℃ under dim light (5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and the luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, USA), according to the manufacturer's instructions. Primers are listed in *SI Appendix* Table S1. Mutations were confirmed by sequencing.

Chromatin immunoprecipitation (ChIP)-qPCR

2-week-old seedlings were harvested and ~2 g of tissue was fixed in 1 % formaldehyde solution and cross-linked under vacuum for 15 min. A final concentration of 0.25 M glycine was used to quench the formaldehyde for 5 min under vacuum. After washing twice with cold deionized water, the tissue was ground in liquid N2 and extraction of chromatin was performed as described previously (14). Prior to immunoprecipitation, 5 μg of anti-GFP antibody were pre-incubated with 20 μl of protein A magnetic beads (Millipore, USA) at 4 ℃ on a rotator overnight. Sonicated chromatin supernatant (250 μl) was diluted to 500 μl and pre-cleared with 20 μl of protein A magnetic beads for 1 h at 4 ℃. Supernatants were incubated with the prepared antibody-bound beads at 4 ℃ for 2 h, and beads were washed sequentially with low-salt wash buffer, high-salt wash buffer, and TE buffer. Elution and reverse cross-linking was performed as previously described (14). The resulting immunoprecipitated DNA was purified with the Qiaquick PCR purification kit (Qiagen, USA), and used for qPCR to examine the enrichment of target genes using the primers listed in *SI Appendix* Table S1.

Chemical induction of *PRR9* in inducible *PRR9-LUC* transgenic plants

For methoxyfenozide (MOF) treatments, inducible *PRR9-LUC* plants were grown on soil under long day conditions. After 3-week, inducible *PRR9-LUC* plants were transferred to constant light (LL) and then grown for 1 more day before the start of the induction experiment. The third and fourth leaves were detached at ZT 1 and floated in 6 well plates supplemented DMSO (mock control) or 50 μM MOF. Samples were collected for gene expression analysis after 12 hours treatment.

Supplementary Figures

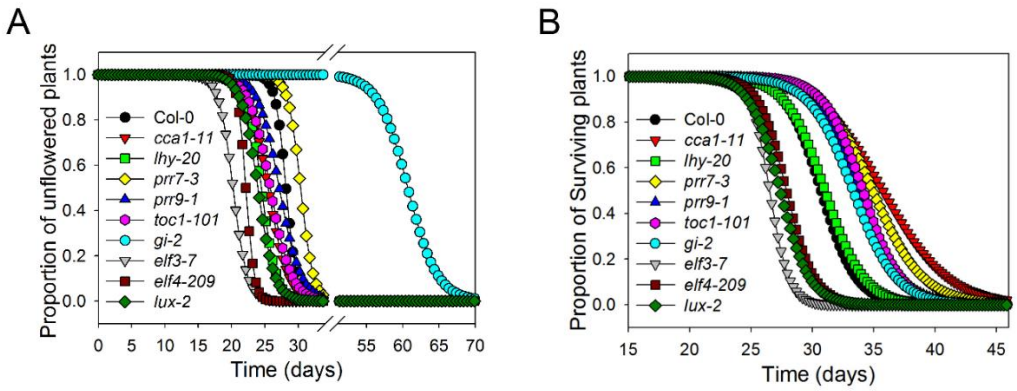

Fig. S1. Regression analysis of flowering and leaf senescence timing in clock mutants. (A) The flowering time of each individual in each mutant population was measured, and the proportion of unflowered plant over time was calculated. This data was regressed to the sigmoidal function below using the optimize function of the Scipy package. Variable k in the function determines the slope of the curve and variable x_0 is the time y reaches 0.5. (B) The proportion of surviving plants in each mutant population was measured. The data was regressed to the sigmoidal function in same way.

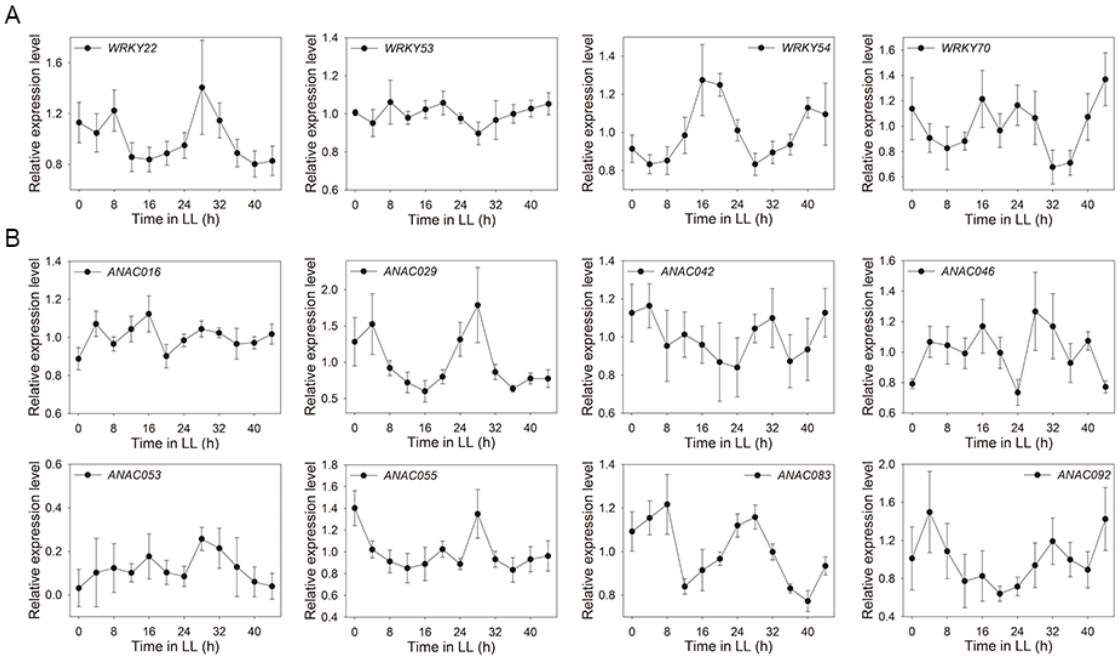

Fig. S2. Expression of senescence-related transcription factor genes from microarray data. (A) *WRKY* family genes from GO term; Aging. (B) *NAC* family genes from GO term; Aging. Microarray data from DIURNAL (http://diurnal.cgrb.oregonstate.edu) are averaged and normalized to the average value over the duration of the experiment to compare relative expression levels among these genes. Error bars indicate SEM. Experimental sets were as follows: LL (LLHC), continuous light (LL) following entrainment to 12 h hot/12 h cold cycles in LL (LLHC); LL (LDHC), LL following entrainment to 12 h light and hot/12 h dark and cold (LDHC); LL12 (LDHH) and LL23 (LDHH), LL following entrainment to 12 h light/12 h dark (LDHH).

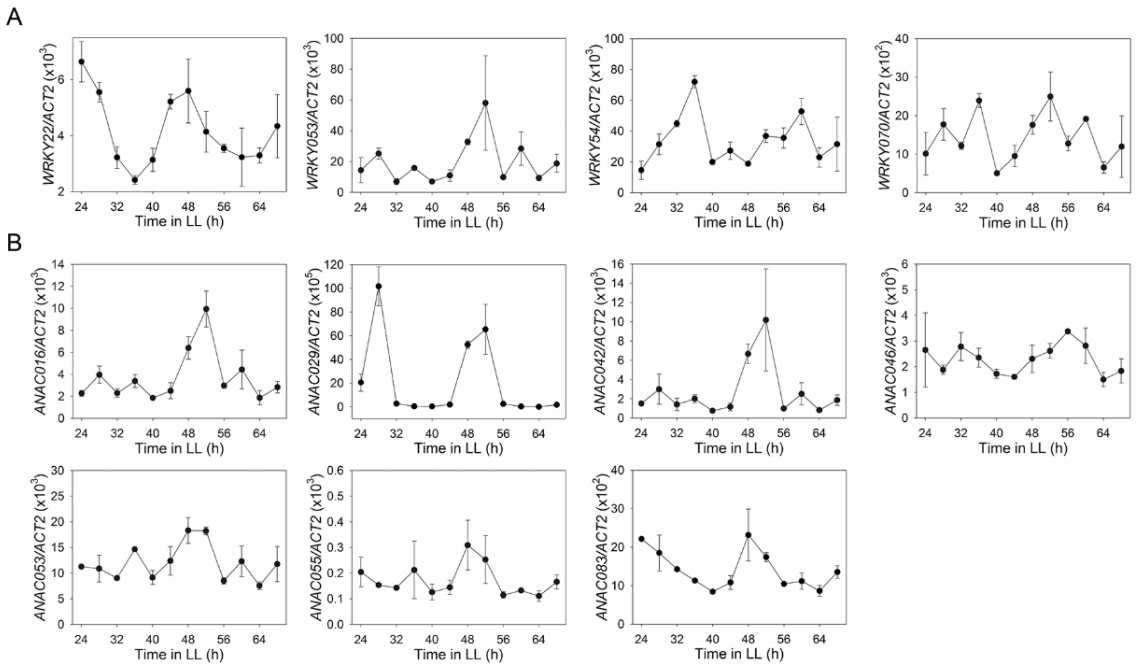

Fig. S3. Expression of senescence-related transcription factor genes is under circadian control. (A and B) Quantitative RT-PCR analysis of clock and age-associated genes. Young (3-week-old) plants grown in LD (16 h light/8 h dark) were transferred to LL and leaves were harvested at indicated times. Expression of WRKY family genes, *WRKY22*, *WRKY54*, and *WRKY70* (A) and NAC family genes, *ANAC016*, *ANAC029*/*NAP*, *ANAC042*, *ANAC046*, *ANAC053*, *ANAC055*, and *ANAC083*/*VNI2* (B). *ACT2*, internal control. Data are presented as the mean ± SEM of biological triplicates.

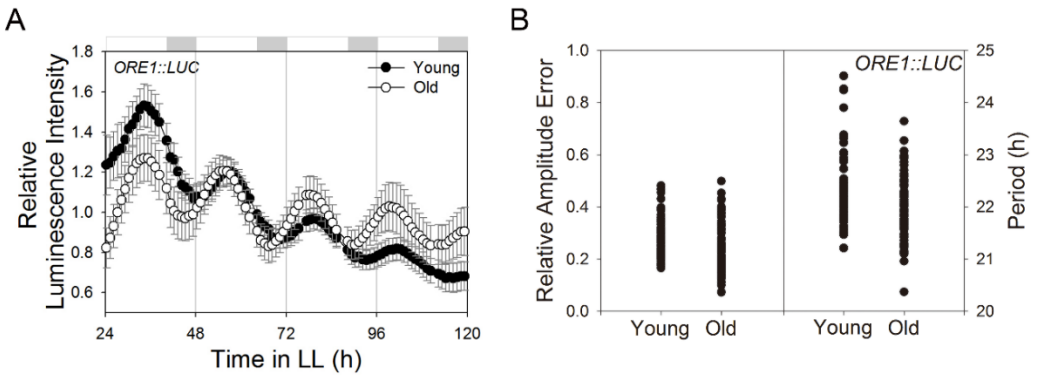

Fig. S4. *ORE1* promoter activities in young and aged leaves. (A) *ORE1* promoter activities under LL in young (3-week-old) and aged (5-week-old) Col-0 leaves. Luminescence intensities from *ORE1:LUC* in excised leaves were measured (mean ± SD, n=48). (B) Comparison of Relative Amplitude Error (RAE) of *ORE1:LUC* expression between young and aged Col-0 leaves (left panel). Comparison of period length of *ORE1:LUC* expression between young and aged Col-0 leaves (right panel).

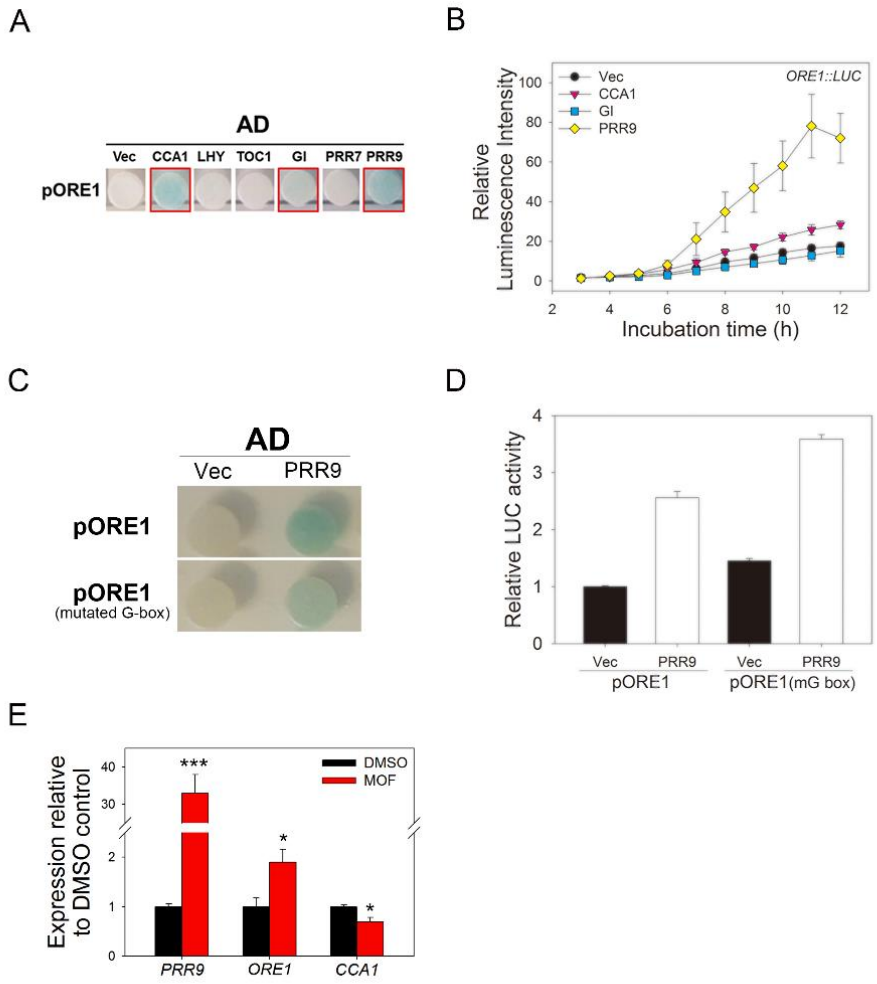

Fig. S5. PRR9 binds to the *ORE1* promoter and induces its transcription. (A) Binding of clock oscillator proteins to the promoter of *ORE1* by yeast one-hybrid assay. An effector plasmid containing one of six clock oscillator genes and a reporter plasmid (*ORE1:lacZ*) were co-transformed into the EGY48 yeast strain. The growth of a blue yeast colony on selective medium containing X-gal indicates a positive interaction. The effector plasmid without clock oscillator genes (vector alone) plus the reporter plasmid served as a negative control. (B) Transactivation assay of *ORE1* promoter by clock oscillator genes using a protoplast system. Protoplasts were co-transfected with the *ORE1:LUC* reporter and an effector plasmid containing one of three clock oscillator genes. Luminescence intensities from *ORE1:LUC* in leaves were measured every hour and relative expression of *ORE1:LUC* was normalized to that of *35Sp:Rluc*, internal control (mean ± SEM, n=6). (C) Binding of PRR9 to the wild-type and mutated promoters of *ORE1* in a yeast one-hybrid assay. An effector plasmid containing the *PRR9* gene and indicated reporter plasmids were co-transformed into the EGY48 yeast strain. (D) Transactivation

assay of *mORE1* promoter with a mutated G box (mG box) by PRR9 using protoplast system (mean ± SEM, n=3). (E) Effect of transient induction of PRR9 on *ORE1* expression. The third and fourth leaves of inducible *PRR9-LUC* transgenic plants were excised and treated with methoxyfenozide (MOF) at ZT1 (*PRR9* peak time) and samples were harvested after 12-h treatment. Comparison of *PRR9*, *ORE1*, and *CCA1* (positive control) transcript levels in mock-treated (DMSO) or treated with 50 μM MOF (mean ± SEM, n=3). Asterisks indicate statistically significant difference from DMSO treatment (*$p<0.05$, ***$p<0.001$; t test).

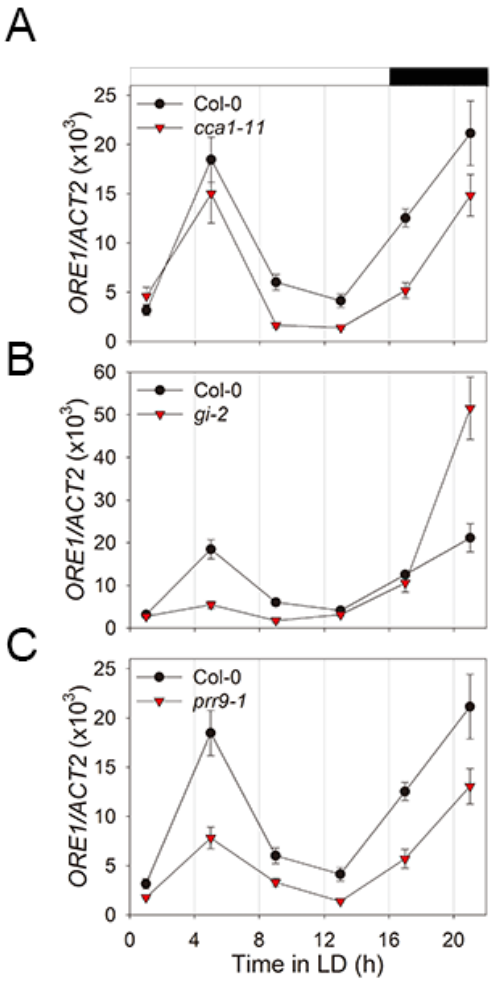

Fig. S6. Diurnal expression of *ORE1* is altered in three clock mutants, *cca1*, *prr9*, and *gi*. (A to C) Young (3-week-old) leaves were harvested under LD (16 h light/8 h dark) at indicated times. Relative abundance of *ORE1* transcript in Col-0 and clock mutants, *cca1-11* (A), *prr9-1* (B), and *gi-2* (C). The third and fourth leaf samples were collected for this analysis. *ORE1* mRNA levels were measured using qRT-PCR and then normalized to *ACT2* expression (mean ± SEM, n=3). Black bar indicates darkness.

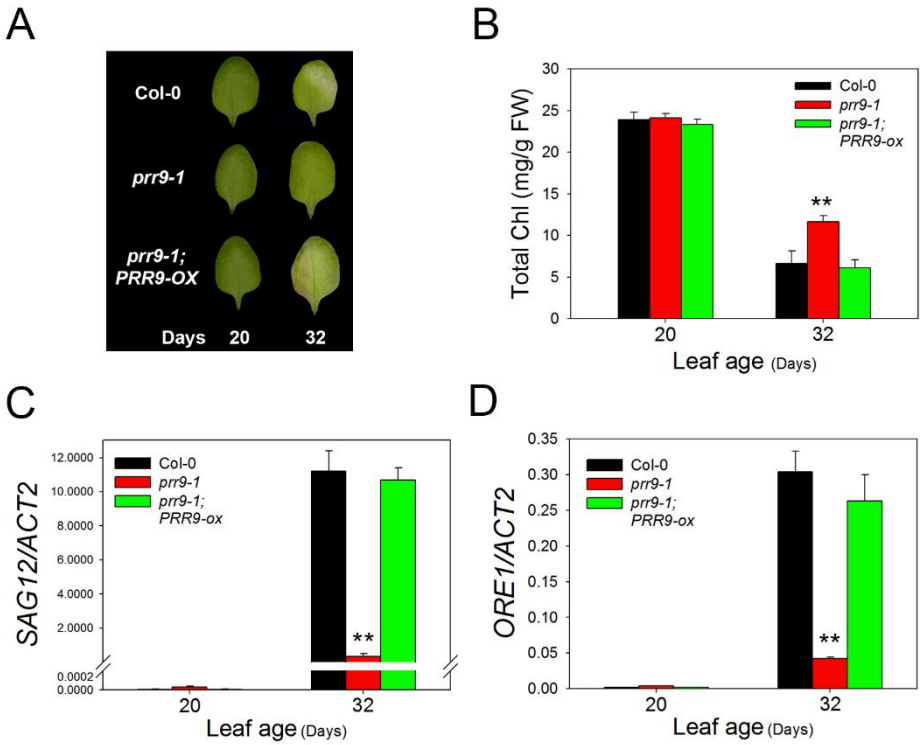

Fig. S7. Overexpression of PRR9 rescues the delayed age-dependent senescence phenotype of *prr9-1*. (A) Chlorophyll loss in Col-0, *prr9-1* and *prr9-1;PRR9-OX*. The photographs show representative of the third and fourth rosette leaves at the indicated days after emergence. (B-D) Analysis of chlorophyll content (B), and mRNA abundance of *SAG12* (C) and *ORE1* (D) in Col-0, *prr9-1*, and *prr9-1;PRR9-OX* at the indicated days (mean ± SEM, n=3). Asterisks indicate statistically significant difference from Col-0 (**p<0.01; t test).

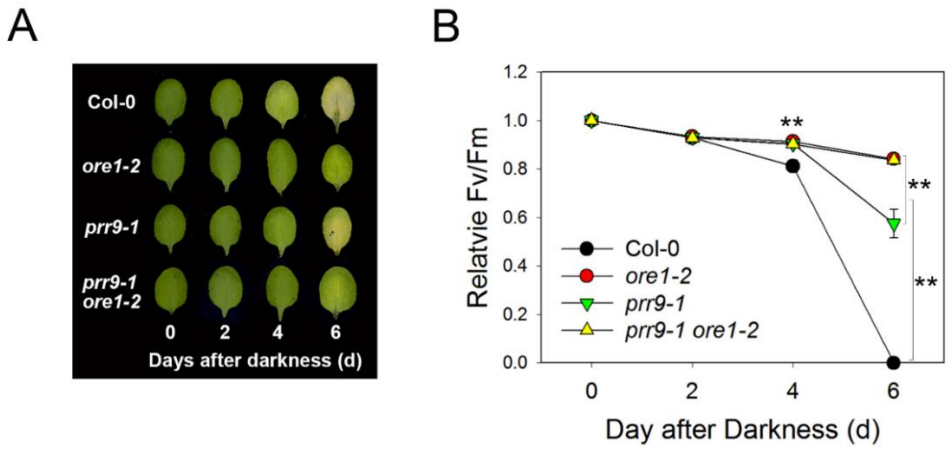

Fig. S8. Dark-induced senescence phenotype in wild type, *ore1-2*, *prr9-1* and *prr9 ore1*. (A) Representative leaves of Col, *ore1-2*, *prr9-1*, and *prr9 ore1* double mutant plants after incubation in darkness for the indicated number of days. (B) Photochemical efficiency of Photosystem II of detached leaves of the indicated genotypes after the indicated days of dark incubation. Data are presented as the mean ± SEM of biological triplicates. Asterisks indicate statistically significant difference from Col-0 (**p<0.01; t test).

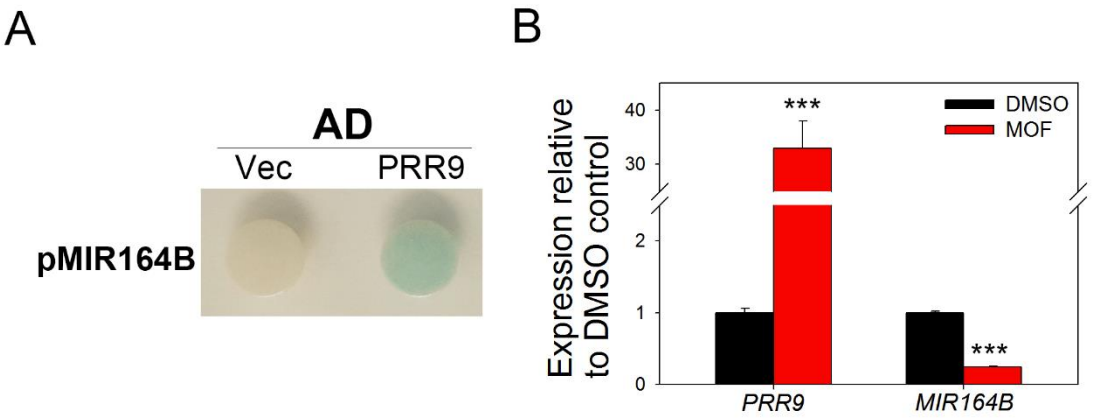

Fig. S9. PRR9 acts as an activator of the *ORE1* promoter and as a repressor of the *MIR164B* promoter. (A) Binding of PRR9 to the promoter of *MIR164B* in a yeast one-hybrid assay. An effector plasmid containing the *PRR9* gene and a reporter plasmid (*MIR164B*:*lacZ*) were co-transformed into the EGY48 yeast strain. (B) Effect of transient induction of PRR9 on *MIR164B* expression. The third and fourth leaves of inducible *PRR9-LUC* transgenic plants were excised and treated with MOF at ZT1 (*PRR9* peak time) and samples were harvested after 12-h treatment. Comparison of *PRR9* and *MIR164B* transcript levels in mock-treated (DMSO) or treated with 50 μM MOF (mean ± SEM, n=3). Asterisks indicate statistically significant difference from DMSO treatment (***p<0.001; t test).

Supplementary Tables

Table S1. Primer list

For real-time PCR.

Gene	**Sequence (5′ → 3′)**	
	Forward	**Reverse**
SAG12	AAAGGAGCTGTGACCCCTATCAA	CCAACAACATCCGCAGCTG
WRKY22	CCAGTGGCTCCAATACCTCT	ATGCCCAGACATCGGAGTT
WRKY53	CGGAAGTCCGAGAAGTGAAG	TCTGACCACTTTGGTAACATCTTT
WRKY54	CACTGCTCAGAACCATGTCAATGC	TGCCTGCGTCTATTGCTGTCAC
WRKY70	TCTCCGTTCTTGATACCTTCG	CGTCGTTATCACATGAAGCATT
ANAC016	ATTCACTTCACAGTCAACAGGTG	GCTGATGAGAACTGGCTCCT
ANAC029	CCGGTACAGACAAAGCCATTCACAG	CCCGAACCAACTAGACTCCGAATCA
ANAC042	ACCAAAACCGATTGGATGAT	TTCTGCAAAGTGTCCATACCTC
ANAC046	GAGCCATGGGATCTACCAAA	GATCCCTCTGGCAGAAAAAGT
ANAC053	GCAACAGAGTTTGAGCCAGA	GCAGGAATAGCACCCAACAT
ANAC055	GCATAAATCACGACGCTCTAAT	GCTCTGCTACTCGTGTATGT
ANAC083	AAGAACCGATTGGATCATGC	AGTGGGACCCATAGAACTCG
ORE1	GTGGGTATGAAGAAAACTTTGG	TTCGTTCTTAGCTGTTTGGGG
MIR164B	ATGAGTTAGTTCTTCATGTGCCCATC	ATATTCCCACGTGCGTAACACTTG
CCA1	CAGCTCCAATATAACCGATCCAT	CAATTCGACCCTCGTCAGACA
PRR9	GTTGAAGAGGAAAGATCGATGCTT	CTGCTCTGGTACCGAACCTTTT
ACT2	CAGTGTCTGGATCGGAGGAT	TGAACAATCGATGGACCTGA

For ChIP assay

Primer	**Sequence (5′ → 3′)**	
	Forward	**Reverse**
Amplicon Ⅰ	CTAGAGTCTTACATTCTCTTTGG	CGTATGTATATGGCTGGAATG
Amplicon Ⅱ	GTAGACACTGGAATAAGACAAG	GTAGACACTGGAATAAGACAAG
Amplicon Ⅲ	GACAACCTCGTATGAACAAA	CCATGTGAAGGTGGTAATG
Amplicon Ⅳ	AGGGAGTTACAGGTGAAG	AGAAGATGCGTAGATTGTTAG
Amplicon Ⅴ	CCATGTAAGTTTCACACCTTATC	GGTCTCTCACACAGAAGAAATA
Amplicon *CCA1*	GGGAGAGATTAACGATC	GGCTACAAAAGAAACTGCC

For site-directed mutagenesis

Primer	**Sequence (5′ → 3′)**	
	Forward	**Reverse**
mG box	CTTTTCATGATCGAATGCTCGATTTTGTTCATACGAGG	CCTCGTATGAACAAAATCGAGCATTCGATCATGAAAAG

For yeast one hybrid assay

Primer	**Sequence (5′ → 3′)**	
	Forward	**Reverse**
miR164B pro	*ACCCCGGGAACCAAAATTTC (Xma I)*	*CTGTCGACTCTTGCTCATCAC (Sal I)*

For luminescence assay

Primer	**Sequence (5′ → 3′)**	
	Forward	**Reverse**
ORE1 pro	*AACTGCAGAGATCGATTGTGTACACGAAGC*	*AGAGGCCTTTTATCCTAATAGGGTTTCTAAAAATG*

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