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

Hyunmin Kim[a], Hyo Jung Kim[a], Quy Thi Vu[b], Sukjoon Jung[b], C. Robertson McClung[c], Sunghyun Hong[a,b,1], and Hong Gil Nam[a,b,1]

[a]Center for Plant Aging Research, Institute for Basic Science (IBS), Daegu 42988, Republic of Korea; [b]Department of New Biology, Daegu Gyeongbuk Institute of Science and Technology (DGIST), Daegu 42988, Republic of Korea; and [c]Department of Biological Sciences, Dartmouth College, Hanover, NH 03755

The circadian clock coordinates the daily cyclic rhythm of numerous biological processes by regulating a large portion of the transcriptome. In animals, the circadian clock is involved in aging and senescence, and circadian disruption by mutations in clock genes frequently accelerates aging. Conversely, aging alters circadian rhythmicity, which causes age-associated physiological alterations. However, interactions between the circadian clock and aging have been rarely studied in plants. Here, we investigated potential roles for the circadian clock in the regulation of leaf senescence in plants. Members of the evening complex in *Arabidopsis* circadian clock, EARLY FLOWERING 3 (ELF3), EARLY FLOWERING 4 (ELF4), and LUX ARRHYTHMO (LUX), as well as the morning component PSEUDO-RESPONSE REGULATOR 9 (PRR9), affect both age-dependent and dark-induced leaf senescence. The circadian clock regulates the expression of several senescence-related transcription factors. In particular, PRR9 binds directly to the promoter of the positive aging regulator *ORESARA1* (*ORE1*) gene to promote its expression. PRR9 also represses *miR164*, a posttranscriptional repressor of *ORE1*. Consistently, genetic analysis revealed that delayed leaf senescence of a *prr9* mutant was rescued by *ORE1* overexpression. Thus, PRR9, a core circadian component, is a key regulator of leaf senescence via positive regulation of *ORE1* through a feed-forward pathway involving posttranscriptional regulation by *miR164* and direct transcriptional regulation. Our results indicate that, in plants, the circadian clock and leaf senescence are intimately interwoven as are the clock and aging in animals.

circadian clock | leaf senescence | PRR9 | ORE1 | miR164

Aging and death are the inevitable fates of organisms. Aging is considered an evolved life history strategy specific to a given species, as each species exhibits its own characteristic life history of aging and death (1). Plants also undergo aging and death in a species-specific manner and show highly diverse life history strategies (2). Annual plants such as rice and *Arabidopsis* show a relatively short and well-defined seasonal lifespan. Trees can live thousands of years; however, their organs age and die, as observed in shedding of autumn leaves (3). The age-dependent senescence of leaves is critical for plants' fitness and productivity. Leaf senescence mobilizes the nutrients accumulated through photosynthesis and nutrient uptake to newly developing leaves or seeds (4). Thus, when and how the aging process or age-dependent senescence of leaf organs proceeds critically affects fitness and productivity at the whole plant level. Plant-specific transcription factor (TF) families such as NAC (NAM, ATAF, and CUC) and WRKY control plant leaf senescence (5), implying that unique regulators of plant-specific TFs may underlie aging in plants.

Most organisms are influenced by the daily and annual cycles of light intensity and duration and temperature (6). Organisms accordingly coordinate their endogenous processes with these environmental cycles to enhance their fitness, leading to the evolution of circadian clocks in many organisms. The circadian clock is an endogenous biological clock that generates ~24-h rhythms, regulating a large fraction of the transcriptome to cope with the daily environmental cycles (7). Plant circadian clocks also regulate many aspects of development and physiology throughout life, such

as flowering (8). Plants are sessile and cannot avoid unfavorable conditions by mobility, so they likely experience greater pressure than animals to cope with daily environmental changes. Circadian clocks in plants have been suggested to function as a "mastermind" of plant life (9), coordinating internal physiological status with environmental cycles. In many systems, the 24-h cyclic rhythm is generated by interconnected feedback loops of the core circadian oscillator.

The physiological effect of aging encompasses the circadian clock system. In animals, aging is associated with the disruption of many circadian rhythms, such as a change of circadian period, delayed recovery from jet lag, and reduced amplitude of clock gene expression (10, 11). Conversely, the circadian clock also protects animals from aging. Disruption of circadian systems accelerates aging and reduces longevity. For example, deficiency of the animal core clock genes *CLOCK* or *BMAL1* accelerated aging and several age-dependent phenotypes, such as cancers, in mice (12, 13). Mutations in *Per1* and *Per2*, interconnected with CLOCK and BMAL1, also show premature aging (14). In plants, however, the interaction of circadian clock and aging has been rarely explored, despite the importance of both processes in plant physiology.

In this study, we reveal regulatory mechanisms that tightly link aging and the circadian clock in plants, using *Arabidopsis* leaf senescence as a model system. We report that several circadian clock genes are required for age-dependent and/or dark-induced leaf senescence. We find that the core clock component PRR9 positively regulates an aging regulator *ORE1*, directly via transcriptional activation and indirectly by suppressing *miR164*, a posttranscriptional repressor of *ORE1*. Importantly, *prr9* mutant plants showed reduced *ORE1* transcript levels and delayed age-induced

Significance

The circadian clock is involved in aging in animals, where mutations in core clock genes accelerate aging. However, little is known about the relationship between aging and the circadian clock in plants. Using the well-studied process of leaf senescence in *Arabidopsis*, a higher plant, as a model for aging, we show that the circadian clock has a critical role in regulating the aging process in plants. Specifically, we show that PSEUDO-RESPONSE REGULATOR 9 (PRR9), a core clock component, positively regulates leaf senescence. *ORESARA 1* (*ORE1*), an aging regulator, is controlled by PRR9 via direct transcriptional activation and indirectly by suppressing *miR164*, a posttranscriptional repressor of *ORE1*, thus forming a coherent feed-forward regulatory loop.

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leaf senescence. *ORE1* overexpression restores age-associated senescence to *prr9* mutant plants. These results suggest that the circadian clock may control leaf senescence by modulating ORE1 amplitude.

Results

Age-Dependent Leaf Senescence and Flowering Time Are Coordinately Controlled by the Circadian Clock. In *Arabidopsis*, the circadian clock that generates the daily cycles includes many core clock components in the "morning" (CCA1, LHY, PRR7, and PRR9) and "evening" (TOC1, GI, ELF3, ELF4, and LUX) loops. To test the interaction between the circadian system and leaf senescence in *Arabidopsis*, we examined leaf senescence, which is physiologically and genetically well defined in *Arabidopsis* leaves (4), in mutants deficient in these nine core circadian clock components. We first examined leaf yellowing, a visible indicator which reflects chloroplast senescence of mesophyll cells (Fig. 1*A*). At 28 d of leaf age, *elf3-7*, *elf4-209*, and *lux-2* showed greater yellowing compared with wild-type Columbia (Col-0). In contrast, other clock mutants (*cca1-1*, *prr7-3*, *prr9-1*, *toc1-101*, and *gi-2*) remained green until 32 d of leaf age, showing delayed senescence compared with wild type. Of the clock mutants tested, only *lhy-20* showed no change in leaf yellowing. Chlorophyll content and survival were consistent with the leaf yellowing phenotypes (Fig. 1 *B* and *C*). Expression of the cysteine protease-encoding *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*), a representative leaf senescence marker in plants, also was positively correlated with the leaf yellowing phenotypes of clock mutants (Fig. 1*D*). The observation that eight of nine circadian clock mutants affected leaf senescence indicates a strong interplay between the circadian clock and aging in plant leaves.

The circadian system controls photoperiodic flowering (8, 15), which can in turn influence age-dependent leaf senescence. Given that age-dependent senescence is controlled by the circadian clock, we examined whether there is a correlated change in flowering and senescence timing in the clock mutants. For this test, we generated survival and flowering curves (*SI Appendix*, Fig. S1) and measured 50% survival and flowering by regression analyses of the curves. We observed a strong correlation between flowering and leaf senescence timing (correlation coefficient $r^2 = 0.554$, $P = 0.021$) except for the *gi-2* mutant which has an extremely late flowering phenotype (Fig. 1*E*). Thus, these two developmental processes may be coupled by the circadian clock in *Arabidopsis*.

The Circadian Clock Regulates Dark-Induced Leaf Senescence. Because age-dependent leaf senescence can be influenced by other developmental processes such as flowering, we aimed to identify clock components that regulate other forms of leaf senescence. Leaf senescence is regulated not only by the internal factor of aging, but also by many external factors, including hormones, pathogen attacks, drought, high salinity, extreme temperature, and darkness (4). Dark-induced senescence displays many similarities to age-dependent senescence, such as chlorophyll and protein degradation (16). However, transcriptomic analysis revealed that age-dependent versus dark-induced senescence triggers significantly different gene expression profiles and signaling pathways (5, 16). To investigate whether the circadian clock regulates dark-induced senescence, we exposed the third and fourth rosette leaves, detached from 3-wk-old wild-type and nine core clock mutant plants, to darkness. We found that *elf3-7*, *elf4-209*, and *lux-2* showed significantly faster dark-induced senescence than wild-type
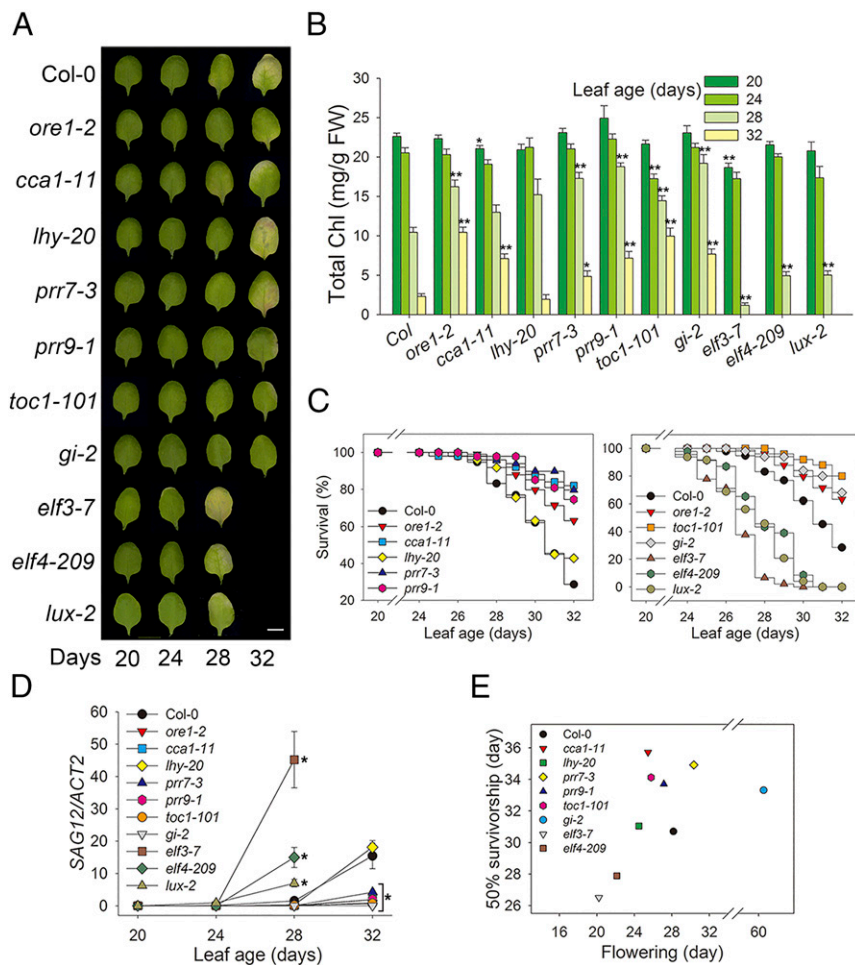

Fig. 1. Circadian clock genes are associated with age-dependent senescence. (*A*) Chlorophyll (Chl) loss in Col-0, *ore1-2* (positive control), and clock mutants. The photographs show representative of the third and fourth rosette leaves at the indicated days after emergence. (Scale bar: 0.5 cm.) (*B*–*D*) Analysis of chlorophyll content (*B*), survivorship (*C*), and expression of age-associated gene (*SAG12*) (*D*) in leaves of the indicated genotypes at the indicated days. (*E*) Association between flowering and senescence in Col-0 and clock mutants. Data are presented as the mean ± SEM of biological triplicates. *$P < 0.05$, **$P < 0.01$; *t* test.
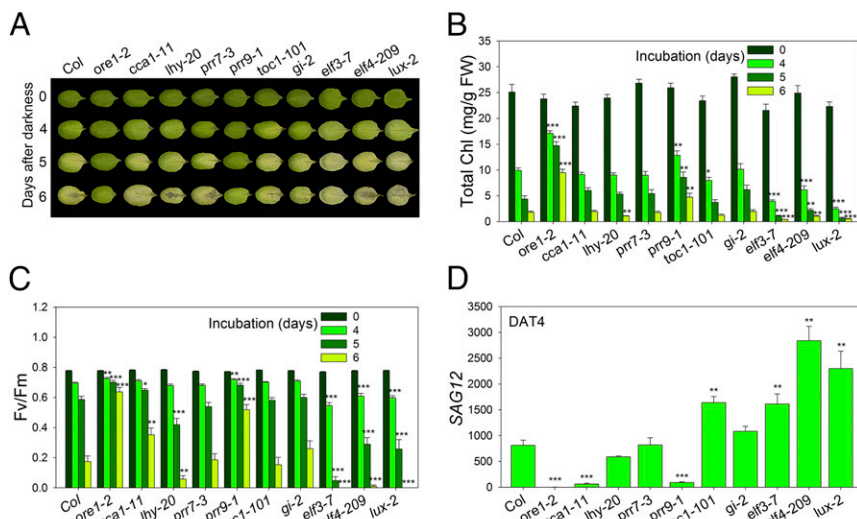
Fig. 2. Dark-induced senescence phenotype of clock mutants in *Arabidopsis*. (*A*) Representative leaves of Col-0, *ore1-2* (positive control), and clock mutant plants after incubation in darkness at the indicated days. (*B* and *C*) Analysis of chlorophyll content (*B*) and photochemical efficiency of PSII (*C*) of detached leaves of the indicated genotypes at the indicated days after dark incubation. (*D*) Expression of the senescence-induced *SAG12* gene at day 4 after dark treatment (DAT4) relative to DAT0 in leaves of the indicated genotypes. Data are presented as the mean ± SEM of biological triplicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; *t* test.

(Col-0), consistent with their effects on age-dependent senescence and with previous findings that *ELF3* suppresses senescence in darkness (17). However, most of clock mutants (*cca1-1*, *lhy-20*, *prr7-3*, *toc1-101*, and *gi-2*) showed similar rates of senescence in darkness. Of the clock mutants tested, only *prr9-1* displayed significantly delayed dark-induced senescence compared with wild type (Fig. 2*A*). Quantitative assays of chlorophyll content, photochemical efficiency, and *SAG12* expression were consistent with the leaf yellowing phenotypes of *prr9-1* (Fig. 2 *B–D*). Thus, *PRR9* promotes both dark-induced and age-dependent leaf senescence.

The Circadian Clock Regulates Several Senescence-Related Transcription Factors. The aging process in *Arabidopsis* is regulated by senescence-related NAC and WRKY transcription factor families, among others (18). Therefore, we tested whether genes in these two families are regulated by the circadian clock to mediate clock-controlled aging. We selected 8 *NAC* and 4 *WRKY* genes associated with the Gene Ontology (GO) term "aging" (19), screened them for potential circadian regulation in the Diurnal database (20), and found that six of them oscillated in a circadian clock-dependent manner (*SI Appendix*, Fig. S2). We also evaluated the expression of all 12 genes by qRT-PCR and confirmed that at least three *NAC* (*ANAC029*, *083*, *092*, also called *ORE1*) and two *WRKY* (*WRKY22*, *54*) genes showed clear circadian expression patterns with distinct amplitudes, phases of peak expression, and waveforms (Fig. 3*A* and *SI Appendix*, Fig. S3). The circadian regulation of 5 of 12 senescence-related transcription factors further supports the circadian clock influence on the aging process.

ORE1 Is Negatively Regulated by the Clock-Controlled *miR164*. *ORE1/ANAC092* is a well-established positive regulator of the aging process that showed a robust circadian expression pattern (Fig. 3*A*). *ORE1* is down-regulated posttranscriptionally by *miR164* and up-regulated transcriptionally by EIN3 during aging through a trifurcate feed-forward pathway, which affects age-dependent cell death (21, 22). We tested whether the circadian clock controls expression of *miR164*, which would in turn negatively regulate expression of *ORE1*. We found that *MIR164B*, the major among three *miR164* isoforms (*miR164a*, *b*, and *c*) showed a robust circadian expression pattern, peaking antiphasic to *ORE1* (Fig. 3*B*); *MIR164B* and *ORE1* showed peak expression at early night and early morning time points, respectively. This observation suggests that antiphasic expression of *miR164* contributes to the circadian expression of *ORE1*. We confirmed that *miR164* negatively controls the amplitude of circadian expression of *ORE1* by using a *mir164abc* triple knockout mutant line and a line overexpressing *miR164B* (*miR164B-ox*). In the *mir164abc* null mutant, the circadian amplitude of *ORE1* mRNA was over

twofold higher than that of wild type (Fig. 3*C*). In contrast, the circadian amplitude of *ORE1* mRNA was greatly (over fivefold) attenuated in the *miR164B-ox* line (Fig. 3*D*). These results demonstrate that the circadian clock negatively regulates the amplitude of *ORE1* mRNA levels via antiphasic expression of *miR164*.

PRR9 Directly Promotes Cyclic Transcription of *ORE1*. *miR164* suppresses the amplitude of *ORE1* mRNA levels, indicating that other mechanisms must promote circadian expression of *ORE1*. To investigate whether *ORE1* promoter activity is under circadian control, we used transgenic reporter plants in which the *ORE1* promoter drives luciferase (LUC) expression. We observed robustly rhythmic luciferase activity in young (3 wk old) and aged (5 wk old) transgenic plants under continuous light (LL) (*SI Appendix*, Fig. S4), revealing that the circadian clock regulates *ORE1* promoter activity.

Most of the circadian core clock components are transcription factors (23). To investigate whether these circadian components
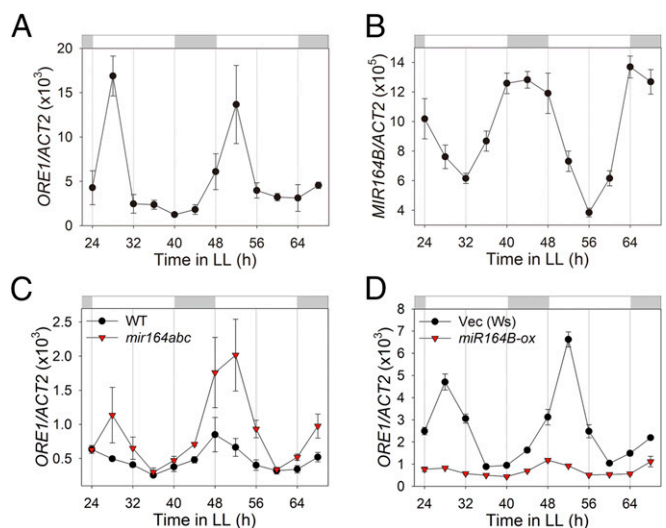

Fig. 3. *ORE1* is posttranscriptionally regulated by clock-controlled *miR164*. (*A* and *B*) mRNA abundance of *ORE1* (*ANAC092*) (*A*) and *MIR164B* (*B*) in young (3 wk old) Col-0 leaves under LL (*n* = 3). (*C*) mRNA abundance of *ORE1* in wild-type and *mir164abc* mutant plants under LL (*n* = 3). (*D*) mRNA abundance of *ORE1* in vector control (Vec) and a *miR164B*-overexpressing line (*miR164B-ox*) in young (3 wk old) leaves under LL (*n* = 3). *ACT2*, internal control. Data are presented as the mean ± SEM of biological triplicates.

regulate the *ORE1* promoter through direct binding, we performed yeast one-hybrid (Y1H) assays. We tested six core clock components, CCA1, LHY, PRR7, PRR9, TOC1, and GI, and found that PRR9, CCA1, and GI bind to the *ORE1* promoter in yeast (*SI Appendix*, Fig. S5A). We then examined the *ORE1* transactivation capacity of these three clock components using an *Arabidopsis* protoplast reporter assay. Among these three, PRR9 activated the *ORE1* promoter in planta twofold or more compared with CCA1, GI, and vector alone (*SI Appendix*, Fig. S5B), suggesting that PRR9 is a direct activator of the *ORE1* promoter. To investigate whether these transcription factors regulate diurnal *ORE1* transcript levels, we monitored the expression of *ORE1* in leaves from *cca1-11*, *prr9-1*, and *gi-2* mutant plants versus wild type. We found that *ORE1* mRNA levels were affected in *prr9-1* and in *gi-2* mutant plants, with the peak expression of *ORE1* mRNA being reduced more than twofold in the mutants compared with that in wild type (*SI Appendix*, Fig. S6). Further, cyclic *ORE1:LUC* activity (Fig. 4 A and B) and overall *ORE1* transcript levels (Fig. 4C) were greatly reduced in the *prr9-1* mutant in the free-running condition of constant light. Together, these data suggest that PRR9 is a direct positive regulator of circadian *ORE1* transcription.

To identify the PRR9-binding domain(s) within the *ORE1* promoter, we generated overexpressed PRR9 transgenic plants driven by the *CsV* promoter in the *prr9-1* mutant to perform chromatin immunoprecipitation (ChIP)-PCR. *CsV:PRR9* (*PRR9-ox*) rescued the delayed age-dependent leaf senescence phenotype of *prr9-1* (*SI Appendix*, Fig. S7). In addition, ChIP-PCR experiments using *prr9; PRR9-ox* plants showed enrichment of PRR9 binding to the *CCA1* promoter (Fig. 4D), consistent with earlier study (24). PRR9 associated with two regions within the *ORE1* promoter (Fig. 4D). PRR9 transcriptionally represses several clock genes, including *CCA1*, by binding to G-box elements. The *ORE1* promoter contains a single G-box element at −1376 bp (Fig. 4D). To test whether the G box in the *ORE1* promoter is associated with transcriptional regulation of *ORE1* by PRR9, we performed yeast one-hybrid assays with mutated G-box element and found that PRR9 binds to

the mutated *ORE1* promoter lacking the G box (*SI Appendix*, Fig. S5C). In a protoplast reporter assay, we found that PRR9-mediated transcriptional induction was about 2.5-fold for both the wild-type and mutated *ORE1* promoters. However, the basal expression level of the mutated *ORE1* promoter was elevated relative to the wild-type promoter (*SI Appendix*, Fig. S5D). Thus, we conclude that PRR9 positively regulates the *ORE1* promoter through a novel mechanism distinct from binding the G-box element.

To further confirm the role of PRR9 on the expression of *ORE1* as a transcriptional activator, we generated *PRR9-LUC* transgenic plants in which this translational fusion of LUC to the C terminus of PRR9 was under the control of an ecdysone agonist-inducible promoter, which allows inducible expression of *PRR9-LUC* with the chemical inducer, methoxyphenozide (MOF) (25). Compared with mock treatment (DMSO), MOF treatment not only decreased *CCA1* transcript level, consistent with the direct repression of *CCA1* expression by PRR9 as shown in a previous study (24), but also increased the *ORE1* transcript level (*SI Appendix*, Fig. S5E). The increase in *ORE1* expression in response to transient induction of *PRR9* supports the notion that PRR9 acts as a direct transcriptional activator of ORE1.

ORE1 Is Epistatic to *PRR9* for Regulating Leaf Senescence. The observation that PRR9 activates *ORE1* transcription is consistent with the delayed leaf senescence phenotype of *prr9-1* mutants (Figs. 1 and 2), as reduced expression of *ORE1* also delayed senescence (Fig. 4C and *SI Appendix*, Fig. S6) (21). To elucidate the genetic relationship between *PRR9* and *ORE1* in controlling leaf senescence, we performed an age-dependent leaf senescence assay. Wild-type leaves displayed yellowing from 28 d of age, whereas *prr9-1* mutant leaves remained green until 32 d of age. Importantly, *ore1-2* and *prr9; ore1* mutant leaves remained green until 36 d of age (Fig. 5A). These distinct leaf yellowing phenotypes were consistent with a quantitative assay of chlorophyll content (Fig. 5B), and with expression of *SAG12* (Fig. 5C). These mutants incubated in the dark behaved the same as the mutants analyzed
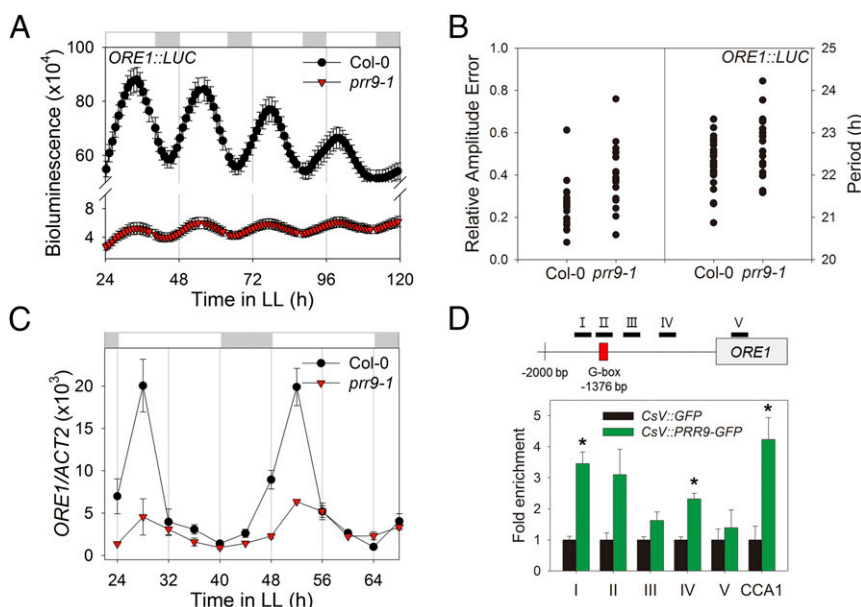

Fig. 4. *ORE1* is directly regulated by PRR9, a core clock component. (A) *ORE1* promoter activity under LL in Col-0 and the *prr9-1* mutant. Luminescence intensity from *ORE1:LUC* in excised leaves (mean ± SEM, n = 24). (B) Relative amplitude error (RAE; a measure of the strength of rhythmicity, where RAE = 0 for a perfect sine wave and RAE = 1.0 defines the limit of a statistically significant oscillation) of *ORE1:LUC* in Col-0 and *prr9-1* mutant was analyzed by fast Fourier transform-nonlinear least-squares (FTT-NLLS) analysis (*Left*, n = 24). Period lengths of *ORE1:LUC* were computed from an experiment in Col-0 and *prr9-1* (*Right*). (C) mRNA abundance of *ORE1* in Col-0 and *prr9-1* (mean ± SEM, n = 3). *ACT2*, internal control. (D) *ORE1* promoter binding affinity of PRR9 in a *CsV:PRR9-GFP* transgenic line. Diagram for the *ORE1* amplicons (I–V) in ChIP assay are indicated. Red box indicates G box (CACGTG). Two-week-old seedlings were harvested 4 h after lights on. The fold enrichment is a ratio of *CsV:PRR9-GFP* normalized to *CsV:GFP* plants. The binding of PRR9-GFP to the *CCA1* promoter is used as a positive control (mean ± SEM, n = 3). Asterisk indicate statistically significant difference from *CsV:GFP* (t test, *P < 0.05).
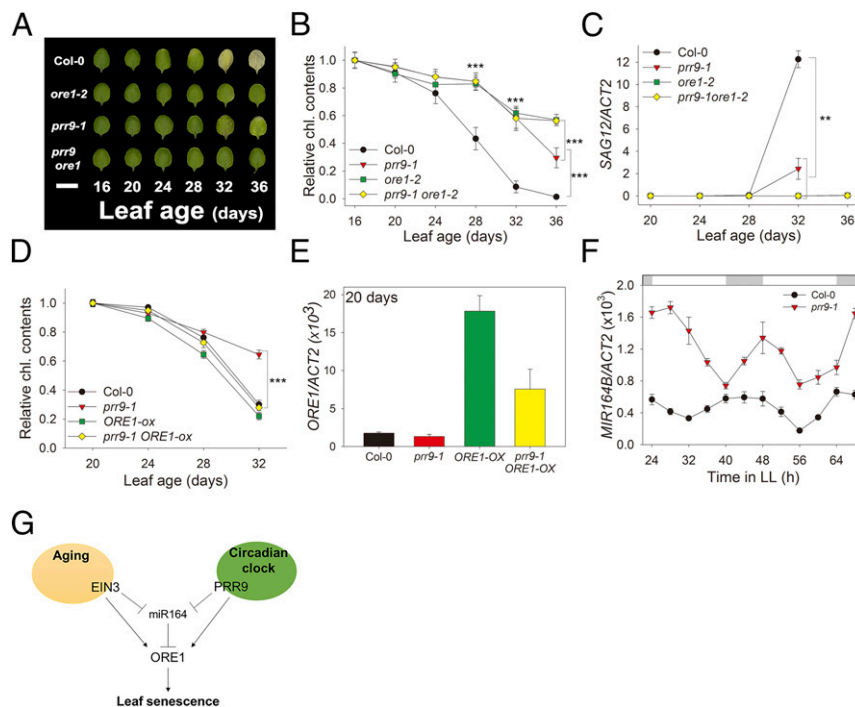
Fig. 5. PRR9 positively regulates leaf senescence via ORE1. (*A*) Chlorophyll loss in Col-0, *ore1-2*, *prr9-1*, and the *prr9-1ore1-2* double mutant. The photographs show representative third and fourth rosette leaves at the indicated days after leaf emergence. (Scale bar: 1 cm.) (*B*) The chlorophyll contents of the indicated genotypes were measured from the third and fourth leaves at indicated days (mean ± SE, *n* = 10). Asterisks indicate statistically significant difference from Col-0 (*t* test, ***P < 0.001). (*C*) The expression of *SAG12* gene in plants of the indicated genotypes at the indicated days (mean ± SEM, *n* = 3). Asterisks indicate statistically significant difference from Col-0 (*t* test, **P < 0.01). (*D*) The chlorophyll content of the third and fourth leaves of the indicated genotypes was measured at indicated days (mean ± SEM, *n* = 8). Asterisks indicate statistically significant difference from Col-0 (*t* test, ***P < 0.001). (*E*) The expression of *ORE1* gene in plants of the indicated genotypes at the indicated days (mean ± SEM, *n* = 3). (*F*) Level of *MIR164B* transcript in Col-0 and *prr9-1* in 20-d-old leaves under LL (mean ± SEM, *n* = 3). ACT2, internal control. (*G*) A trifurcate feed-forward pathway model for regulating leaf senescence by aging and circadian clock. PRR9 positively regulates *ORE1* with a circadian rhythm at both the transcriptional and posttranscriptional levels. Posttranscriptional repression by clock-controlled *miR164* negatively regulates *ORE1* mRNA level. Aging activates *ORE1* expression by a similar trifurcate feed-forward pathway; direct binding of EIN3 to the *ORE1* and *miR164* promoters activates *ORE1* expression directly and indirectly, via repression of *miR164*, a repressor of ORE1, to promote age-dependent senescence.

during age-dependent senescence (*SI Appendix*, Fig. S8), suggesting that PRR9 mediates age-dependent and dark-induced leaf senescence primarily through *ORE1*. To investigate whether PRR9 activates *ORE1* to mediate senescence phenotypes, we tested whether *ORE1* overexpression could rescue *prr9* mutant phenotypes. Indeed, *prr9; ORE1-ox* plant leaves showed age-dependent senescence similar to wild-type and *ORE1-ox* plant leaves, revealing that *ORE1* is epistatic to *PRR9* for controlling leaf senescence (Fig. 5D).

PRR9 Indirectly Regulates *ORE1* Expression via *miR164*. Interestingly, we found that *ORE1* transcript levels were reduced in 20-d-old *prr9; ORE1-ox* leaves compared with *ORE1-ox* leaves (Fig. 5E), suggesting that PRR9 also regulates *ORE1* transcript abundance independently of direct transcriptional activation, possibly at the posttranscriptional level. Since expression of *miR164* is also controlled by the circadian clock, we measured *MIR164B* transcript levels in young (3 wk old) wild-type and *prr9* mutant leaves in LL. The amplitude of *MIR164B* was dramatically increased in the *prr9* mutant compared with wild type (Fig. 5F), consistent with PRR9 functioning as a negative regulator of *miR164* expression.

To test whether PRR9 regulates *miR164* expression directly, we performed a Y1H assay and found that PRR9 binds to the *MIR164B* promoter region directly (*SI Appendix*, Fig. S9A). To evaluate the effect of PRR9 on the expression of *miR164*, we used inducible *PRR9-LUC* plants. In the presence of MOF, *MIR164B* transcript level was decreased (*SI Appendix*, Fig. S9B), indicating that PRR9 functions as a direct transcriptional repressor of *miR164B*. Collectively, these data suggest that PRR9 tightly regulates leaf senescence by dual control of *ORE1*, directly at the level of transcriptional

activation of *ORE1* and indirectly at the posttranscriptional level, via direct suppression of *miR164*, a repressor of *ORE1*. This coherent feed-forward loop between a circadian clock component and an aging regulator coordinates leaf senescence.

Discussion

In this report, we investigated the molecular interactions between the circadian clock and aging in *Arabidopsis* leaves. Several of the core circadian components affect age-dependent leaf senescence (Fig. 1). Age-dependent leaf senescence can be influenced by developmental processes such as flowering. Indeed, we found that the age-dependent leaf senescence phenotypes of clock mutants showed significant correlation with flowering time, as expected since the circadian clock controls photoperiodic flowering (15, 26). To identify clock components directly involved in senescence, independent of flowering, we evaluated the effect of clock mutants on dark-induced senescence. Three evening complex (EC) mutants (*elf3*, *elf4*, and *lux*) showed significantly early senescence in darkness, whereas *prr9* mutants showed significantly delayed senescence under these conditions (Fig. 2). Because the evening complex is a known repressor of *PRR9* expression (27, 28), we attribute the early senescence of EC mutants at least in part to a relief of inhibition of *PRR9* and consequent induction of *ORE1*. As plant aging and senescence are highly programmed processes that recycle nutrients to sink sources such as developing seeds, positive regulation of senescence by the circadian clock may be essential for fitness in plants.

Circadian regulation of several plant-specific senescence-related transcription factors (Fig. 3 and *SI Appendix*, Fig. S2)

raised the possibility that clock components directly regulate their expression. We found that *ORE1* promoter activity and transcript levels show circadian rhythmic patterns (Figs. 3 and 4). *ORE1* is posttranscriptionally regulated by *miR164* (21). *miRNAs* also regulate the circadian system in animals, where expression of various *miRNAs* is clock regulated, mediating the circadian clock system by regulating core clocks, inputs, and outputs (29, 30). In *Arabidopsis*, several *miRNAs* (*miR167*, *miR168*, *miR171*, and *miR398*) show rhythmic expression patterns in diurnal conditions, but do not appear to be directly regulated by the circadian clock (31). Here, we showed that *miR164* is under circadian regulation, is expressed in an antiphasic manner relative to its target, *ORE1*, and negatively regulates *ORE1* (Fig. 3). Posttranscriptional regulation via *miRNA* in the circadian clock system thus represents a general regulatory mechanism in both plants and animals. Circadian expression of *ORE1* is negatively regulated by clock-controlled *miR164* at the posttranscriptional level, but positively regulated by PRR9, a core clock component, at the transcriptional level. Moreover, *MIR164B* levels were negatively regulated by PRR9 (Fig. 5*F* and *SI Appendix,* Fig. S9*B*). This regulatory mechanism has identical characteristics to the trifurcate feed-forward pathway for regulation of age-dependent senescence in *Arabidopsis* (Fig. 5*G*). EIN3 induces *ORE1* in an age-dependent manner and simultaneously suppresses the expression of *miR164*, which negatively regulates *ORE1* (21, 22). In this model, the circadian system activates expression of a key positive regulator of aging, *ORE1*, through a core circadian transcription factor, PRR9, thereby affecting the aging process (Fig. 5). *ORE1* is under circadian control but its overall expression level increases with leaf age. On the other hand, *ORE1* still shows a circadian expression pattern in the absence of *PRR9* (Fig. 4 *A* and *C*), suggesting that other clock components contribute to *ORE1* regulation. Recent studies have proposed other trifurcate feed-forward pathways for regulating leaf senescence in *Arabidopsis* (32, 33), suggesting that this regulation may be required to fine tune leaf senescence.

Materials and Methods

Plant Materials and Growth Condition. All clock mutants used in this study: *cca1-11*, *lhy-20*, *prr7-3*, *prr9-1*, and *toc1-101* (34), *gi-2* and *elf4-209* (35), and *elf3-7* (36) are in the Columbia (Col-0) background. The *ore1-2*, *mir164abc*, and *miR164B-ox* line were described previously (21). To generate the *ORE1: LUC* transgenic line, a 2.4-kb *ORE1* promoter was cloned into the gZPXo-megaLUC vector (37) to fuse with the firefly luciferase gene and it was introduced into Col-0 plants by *Agrobacterium tumefaciens*-mediated transformation. All plants expressing luciferase were generated by genetic crossing. See *SI Appendix, SI Materials and Methods* for complete details.

Assay of Age-Dependent Senescence. Leaf senescence was assayed as described previously with minor modifications (21). Chlorophyll was extracted from individual leaves by heating in 95% ethanol at 80 °C. The chlorophyll concentration per fresh weight of leaf tissue was calculated as described previously (38). Survival of the leaves in clock mutants was performed with leaf age; leaves with 50% of leaf area yellowed were counted as senesced. See *SI Appendix, SI Materials and Methods* for complete details.

Details of the experimental programs for analysis of gene expression, luminescence assay, yeast one-hybrid assay, transient expression assay, ChIP-qPCR assay, and chemical induction of PRR9 in inducible *PRR9-LUC* transgenic plants are provided in *SI Appendix, SI Materials and Methods*. The primers used in this study are listed in *SI Appendix,* Table S1.

1. Jones OR, et al. (2014) Diversity of ageing across the tree of life. *Nature* 505:169–173.
2. Adler PB, et al. (2014) Functional traits explain variation in plant life history strategies. *Proc Natl Acad Sci USA* 111:740–745.
3. Guarente L, Ruvkun G, Amasino R (1998) Aging, life span, and senescence. *Proc Natl Acad Sci USA* 95:11034–11036.
4. Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. *Annu Rev Plant Biol* 58:115–136.
5. Breeze E, et al. (2011) High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation. *Plant Cell* 23:873–894.
6. Merrow M, Spoelstra K, Roenneberg T (2005) The circadian cycle: Daily rhythms from behaviour to genes. *EMBO Rep* 6:930–935.
7. Dunlap JC (1999) Molecular bases for circadian clocks. *Cell* 96:271–290.
8. de Montaigu A, Tóth R, Coupland G (2010) Plant development goes like clockwork. *Trends Genet* 26:296–306.
9. Sanchez SE, Kay SA (2016) The plant circadian clock: From a simple timekeeper to a complex developmental manager. *Cold Spring Harb Perspect Biol* 8:a027748.
10. Monk TH (2005) Aging human circadian rhythms: Conventional wisdom may not always be right. *J Biol Rhythms* 20:366–374.
11. Hofman MA, Swaab DF (2006) Living by the clock: The circadian pacemaker in older people. *Ageing Res Rev* 5:33–51.
12. Dubrovsky YV, Samsa WE, Kondratov RV (2010) Deficiency of circadian protein CLOCK reduces lifespan and increases age-related cataract development in mice. *Aging (Albany NY)* 2:936–944.
13. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes Dev* 20:1868–1873.
14. Lee CC (2005) The circadian clock and tumor suppression by mammalian period genes. *Methods Enzymol* 393:852–861.
15. Putterill J (2001) Flowering in time: Genes controlling photoperiodic flowering in Arabidopsis. *Philos Trans R Soc Lond B Biol Sci* 356:1761–1767.
16. Buchanan-Wollaston V, et al. (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. *Plant J* 42:567–585.
17. Sakuraba Y, et al. (2014) Phytochrome-interacting transcription factors PIF4 and PIF5 induce leaf senescence in Arabidopsis. *Nat Commun* 5:4636.
18. Woo HR, Kim HJ, Nam HG, Lim PO (2013) Plant leaf senescence and death–Regulation by multiple layers of control and implications for aging in general. *J Cell Sci* 126:4823–4833.
19. Carbon S, et al.; AmiGO Hub; Web Presence Working Group (2009) AmiGO: Online access to ontology and annotation data. *Bioinformatics* 25:288–289.
20. Mockler TC, et al. (2007) The DIURNAL project: DIURNAL and circadian expression profiling, model-based pattern matching, and promoter analysis. *Cold Spring Harb Symp Quant Biol* 72:353–363.

21. Kim JH, et al. (2009) Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. *Science* 323:1053–1057.
22. Kim HJ, et al. (2014) Gene regulatory cascade of senescence-associated NAC transcription factors activated by ETHYLENE-INSENSITIVE2-mediated leaf senescence signalling in Arabidopsis. *J Exp Bot* 65:4023–4036.
23. Nohales MA, Kay SA (2016) Molecular mechanisms at the core of the plant circadian oscillator. *Nat Struct Mol Biol* 23:1061–1069.
24. Nakamichi N, et al. (2010) PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. *Plant Cell* 22:594–605.
25. Koo JC, Asurmendi S, Bick J, Woodford-Thomas T, Beachy RN (2004) Ecdysone agonist-inducible expression of a coat protein gene from tobacco mosaic virus confers viral resistance in transgenic Arabidopsis. *Plant J* 37:439–448.
26. Song YH, Shim JS, Kinmonth-Schultz HA, Imaizumi T (2015) Photoperiodic flowering: Time measurement mechanisms in leaves. *Annu Rev Plant Biol* 66:441–464.
27. Helfer A, et al. (2011) LUX ARRHYTHMO encodes a nighttime repressor of circadian gene expression in the Arabidopsis core clock. *Curr Biol* 21:126–133.
28. Dixon LE, et al. (2011) Temporal repression of core circadian genes is mediated through EARLY FLOWERING 3 in Arabidopsis. *Curr Biol* 21:120–125.
29. Wang X, Tian G, Li Z, Zheng L (2015) The crosstalk between miRNA and mammalian circadian clock. *Curr Med Chem* 22:1582–1588.
30. Mehta N, Cheng HY (2013) Micro-managing the circadian clock: The role of microRNAs in biological timekeeping. *J Mol Biol* 425:3609–3624.
31. Siré C, Moreno AB, Garcia-Chapa M, López-Moya JJ, San Segundo B (2009) Diurnal oscillation in the accumulation of Arabidopsis microRNAs, miR167, miR168, miR171 and miR398. *FEBS Lett* 583:1039–1044.
32. Sakuraba Y, Kim YS, Han SH, Lee BD, Paek NC (2015) The Arabidopsis transcription factor NAC016 promotes drought stress responses by repressing AREB1 transcription through a trifurcate feed-forward regulatory loop involving NAP. *Plant Cell* 27:1771–1787.
33. Song Y, et al. (2014) Age-triggered and dark-induced leaf senescence require the bHLH transcription factors PIF3, 4, and 5. *Mol Plant* 7:1776–1787.
34. Kim H, Kim Y, Yeom M, Lim J, Nam HG (2016) Age-associated circadian period changes in Arabidopsis leaves. *J Exp Bot* 67:2665–2673.
35. Kim Y, et al. (2012) GIGANTEA and EARLY FLOWERING 4 in Arabidopsis exhibit differential phase-specific genetic influences over a diurnal cycle. *Mol Plant* 5:678–687.
36. McWatters HG, Bastow RM, Hall A, Millar AJ (2000) The ELF3 zeitnehmer regulates light signalling in the circadian clock. *Nature* 408:716–720.
37. Schultz TF, Kiyosue T, Yanovsky M, Wada M, Kay SA (2001) A role for LKP2 in the circadian clock of Arabidopsis. *Plant Cell* 13:2659–2670.
38. Lichtenthaler HK (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods Enzymol* 148:350–382.