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Journal

Proceedings of the National Academy of Sciences of the United States of America, 115(27)

ISSN

0027-8424

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Publication Date

2018-07-03

DOI

10.1073/pnas.1805524115

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Multiple feedback loops of the Arabidopsis circadian clock provide rhythmic robustness across environmental conditions

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Edited by Susan S. Golden, University of California, San Diego, La Jolla, CA, and approved May 29, 2018 (received for review March 30, 2018)

Although circadian oscillators in diverse eukaryotes all depend on interlinked transcriptional feedback loops, specific components are not conserved across higher taxa. Moreover, the circadian network in the model plant *Arabidopsis thaliana* is notably more complex than those found in animals and fungi. Here, we combine mathematical modeling and experimental approaches to investigate the functions of two classes of Myb-like transcription factors that antagonistically regulate common target genes. Both CCA1/LHY- and RVE8-clade factors bind directly to the same *cis*-element, but the former proteins act primarily as repressors, while the latter act primarily as activators of gene expression. We find that simulation of either type of loss-of-function mutant recapitulates clock phenotypes previously reported in mutant plants, while simulated simultaneous loss of both type of factors largely rescues circadian phase at the expense of rhythmic amplitude. In accord with this prediction, we find that plants mutant for both activator- and repressor-type Mybs have near-normal circadian phase and period but reduced rhythmic amplitude. Although these mutants exhibit robust rhythms when grown at mild temperatures, they are largely arrhythmic at physiologically relevant but nonoptimal temperatures. LHY- and RVE8-type Mybs are found in separate clades across the land plant lineage and even in some unicellular green algae, suggesting that they both may have functioned in even the earliest arising plant circadian oscillators. Our data suggest that the complexity of the plant circadian network may have arisen to provide rhythmic robustness across the range of environmental extremes to which plants, as sessile organisms, are regularly subjected.

circadian clock | transcriptional regulation | temperature compensation | feedback loops | modeling

Circadian clocks are found in most eukaryotes and some prokaryotes. Their ubiquity suggests that they are biologically important, and indeed, experiments in plants, mammals, and bacteria have shown that circadian clocks that run with a period matching that of environmental cycles provide a competitive advantage (1–3). Circadian clocks have a complex relationship with the environment, with environmental variables, such as light and temperature, acting as cues to reset the phase of the clock but with clock pace being relatively unchanged across a large range of temperatures and light levels. Plants, as poikilothermic, autotrophic, and sessile organisms, might be expected to have clocks that are particularly sensitive to environmental variation. However, the plant clock is remarkably robust, with a recent study in field-grown rice showing variations in the phase of clock-related gene expression of only 22 min across an entire season (4). This robustness of clock function is also observed in controlled environments, with high-amplitude circadian rhythms persisting in *Arabidopsis thaliana* plants grown under a wide range of light conditions and at temperatures ranging from 12 °C to 32 °C (5–7). Plant rhythms seem more resilient to low temperatures than those of other poikilothermic organisms; for example, circadian rhythmicity is lost in *Drosophila melanogaster*, *Neurospora crassa*, and the dinoflagellate *Lingulodnium polyedrum* (formerly known as Gonyaulax) at temperatures below ∼15 °C to 16 °C (8–10).

Although circadian oscillators in diverse eukaryotes all depend on interlinked transcriptional feedback loops, clockwork components are not conserved across higher taxa. Our current understanding of the plant clock network includes over 20 transcription factors connected in a bewildering number of feedback loops (11, 12). A simplified cycle of the plant circadian network is as follows: around dawn, the Myb-like factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LONG ELONGATED HYPOCOTYL (LHY) repress gene expression via binding to the evening element (EE) motif, a *cis*-element found in the promoters of both core clock and output genes. Target clock genes include *PSEUDO-RESPONSE REGULATOR 5* (*PRR5*), *PRR7*, and *PRR9* and their homolog *TIMING OF CAB EXPRESSION 1* (*TOC1*; also known as *PRR1*). In turn, these day-phased PRR proteins repress *CCA1* and *LHY* to restrict their expression to the late night and early morning. CCA1 and LHY also repress expression of genes that encode members of the evening complex (EC), a trimer of LUX ARRHYTHMO (LUX), EARLY FLOWERING 3 (ELF3), and ELF4. The EC represses expression of *PRR7* and *PRR9*, thus indirectly relieving repression of *CCA1* and *LHY*. Rare positive clock regulators of gene expression include the Myb-like REVEILLE (RVE) factors, which have very similar DNA binding specificity as CCA1 and LHY. In the afternoon, RVE8 and probably its

Significance

While all eukaryotic circadian clocks depend on transcriptional feedback loops, the plant circadian network is uniquely complex. An apparent redundancy in the plant clock is two types of transcription factors that antagonistically regulate common target genes. Here, we show that, although loss of either repressor- or activator-type factors perturbs clock pace and plant development, simultaneous loss of both types of factors restores near–wild-type development and largely rescues circadian period and phase phenotypes at ambient temperatures. However, in the higher-order mutant, rhythmic amplitude is reduced under optimal temperature, and rhythmicity is lost at low or high temperatures within the physiologically relevant range. Our data suggest that the multiple feedback loops of the plant clock help ensure rhythmicity under adverse environmental conditions.

Author contributions: A.S.-K. and S.L.H. designed research; A.S.-K. and R.W.K. performed research; V.F. contributed new reagents/analytic tools; A.S.-K., R.W.K., and S.L.H. analyzed data; and A.S.-K. and S.L.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1805524115/-/DCSupplemental.

Published online June 18, 2018.

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homologs RVE4 and RVE6 bind to EE motifs to activate expression of *PRR* and *EC* genes. PRR proteins, in turn, repress *RVE8* expression, forming yet another transcriptional feedback loop (11, 12) (Fig. 1*A*).

In contrast to the intricacy seen in even this simplified view of the plant circadian oscillator, clock networks in fungi and animals are composed of only a handful of transcription factors connected by one or a few feedback loops (13). To examine circadian function in plants with a simplified clock network, we have obtained plants simultaneously mutant for genes in the antagonistic LHY and RVE8 transcription factor clades. While mutation of either type of factor causes growth and circadian phenotypes, simultaneous loss of both largely restores growth and circadian phenotypes when plants are maintained at mild temperatures. However, clock function at low and high temperatures is severely compromised in these higher-order mutants. These data suggest that the complexity of the plant circadian oscillator may be needed to promote robust rhythmicity across the wide range of environmental conditions to which plants are frequently subjected.

Results

Modeling the Effects of Altered Levels of Activators and Repressors on Plant Clock Function. The opposite circadian phenotypes seen in *Arabidopsis* plants mutant for *LHY*-type or *RVE8*-type Myb-like transcription factors (14–18) suggest that they have antagonistic functions in the clock network. Previous in silico analysis of mammalian clock components has suggested that the coordinate action of repressors and activators helps control the circadian phase and amplitude of common targets genes (19). We hypothesized that LHY-type and RVE8-type factors, acting as repressors and activators, respectively, of shared targets, might play a similar role in the plant circadian network. Since nonclock-regulated activators of gene expression are thought to play an important role in the plant clock network (20), we modified the mathematical expression developed by Ueda et al. (19) to include a noncycling activator of gene expression in addition to the cycling activator and repressor. In our model, we combined CCA1 and LHY activity as one cycling repressor component (termed "LHY") and members of the RVE8 subclade as one cycling activator component (termed "RVE8") (Fig. 1*A*), and we assumed equal rhythmic amplitude and regulatory potency for both. Times of peak regulatory activities were assigned based on the reported peak phases of LHY and RVE8 protein accumulation (17, 21, 22) (Fig. 1*B*). This simple model predicts that genes coordinately regulated by LHY- and RVE8-type factors would have peak expression near dusk (Fig. 1*C*), consistent with the phases of the transcripts of most genes identified as RVE8 or CCA1 targets in genome-wide studies (18, 23, 24).

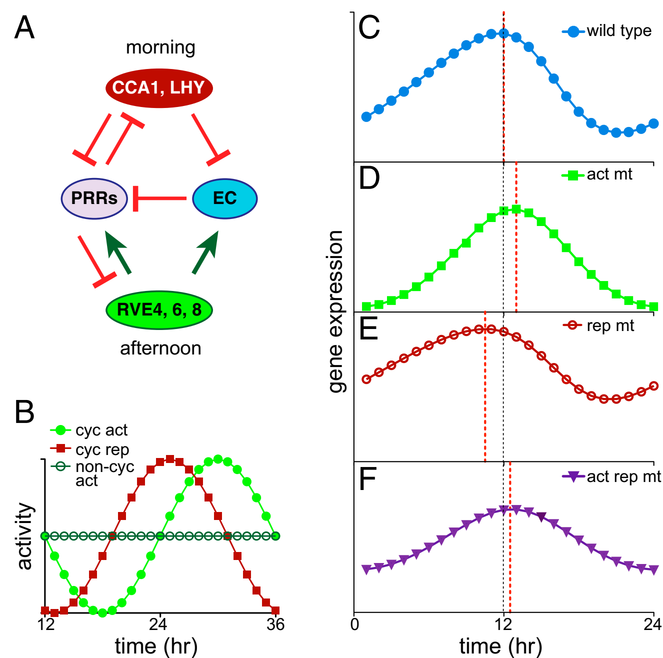

Fig. 1. In silico model of effects of antagonistic transcription factors on circadian phase and amplitude of a common target gene. (*A*) Simplified cartoon of the plant clock. Red bars indicate transcriptional repression, and green arrows indicate transcriptional activation. Details are in the text. (*B–F*) In silico model of clock-regulated expression of a gene directly regulated by antagonistic transcription factors. Time 0 = dawn. (*B*) Simulated times of action of a clock-regulated transcriptional activator (cycling activator, cyc act; light green), repressor (cycling repressor, cyc rep; brown), and a constitutive activator (noncycling activator, non-cyc act; dark green). (*C*) Expression pattern of a gene regulated by the three factors depicted in *B*. (*D–F*) Expression of the target gene on simulated reduction of function of (*D*) the cycling activator (activator mutant, act mt), (*E*) the repressor (cycling repressor mutant, rep mt), or (*F*) both the activator and the repressor (cycling activator and repressor mutant, act rep mt). Vertical red lines indicate peak phases.

We next simulated an *RVE8* loss-of-function mutant by reducing the strength of the cycling activator in the model, which caused a later peak and lower trough for the hypothetical target gene (Fig. 1*D*), consistent with published expression patterns of RVE8 targets, such as *TOC1*, in the *rve468* mutant (18). Simulation of an *LHY* reduction-of-function mutant resulted in an earlier peak phase and higher trough level (Fig. 1*E*), recapitulating experimental results observed in *cca1 lhy* double mutants (15). Finally, simulation of a *cca1 lhy rve468* mutant by reducing the strength of both the cycling activator and the repressor resulted in output gene expression with a near–wild-type peak phase but reduced amplitude (Fig. 1*F*). This simulation suggests that near-normal circadian phase can be achieved in Myb mutants as long as the activity of the positive and negative factors are balanced but that lower overall expression levels of the Mybs might compromise rhythmic amplitude.

Removal of both Repressors and Activators Rescues Growth and Flowering Phenotypes. To test the hypothesis that simultaneous reduction of function of *LHY*-clade and *RVE8*-clade genes might rescue phenotypes seen in plants mutant for one or the other type of factor, we compared the phenotypes of *cca1-1 lhy-20* (25) (hereafter *cca1lhy*), *rve4-1 rve6-1 rve8-1* (18) (hereafter *rve468*), and *cca1-1 lhy-20 rve4-1 rve6-1 rve8-1* (hereafter *quint*). qRT-PCR analysis of the quintuple mutant revealed that no *CCA1* or *RVE4* transcripts could be detected but that *RVE8* is expressed at low levels and *RVE6* is expressed at about one-half the level seen in the wild type (*SI Appendix*, Fig. S1). *LHY* is also detectably expressed in both the *cca1lhy* and *quint* mutants, with peak levels ∼30% of the wild type (*SI Appendix*, Fig. S1). Therefore, these mutants represent loss of function but not complete null alleles for *LHY*-type and *RVE8*-type genes.

Previous studies reported numerous phenotypes for *cca1lhy* mutants, including early flowering, and short hypocotyls (15, 26). In contrast, *rve468* triple-mutant plants display delayed flowering, elongated hypocotyls, and larger rosettes (due to both longer petioles and larger leaf blades) than the wild type (27). We found that these growth and flowering phenotypes are largely alleviated in the *quint* mutants. While the petioles of *cca1lhy* plants are shorter and those of *rve468* are longer than the wild type, the petioles of *quint* mutant plants are not significantly different from the wild type in long days (LDs; 16 h light, 8 h dark) and are closer to the wild type than either parental mutant in short days (8 h light, 16 h dark) (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). Similarly, while *cca1lhy* has shorter (26) and *rve468* has

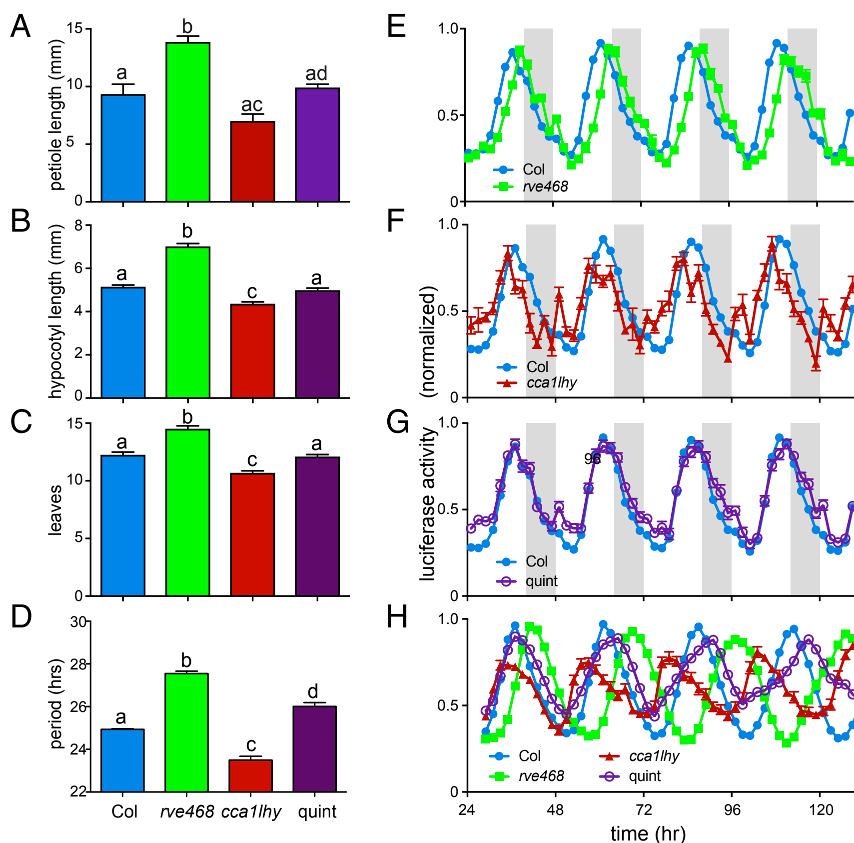

Fig. 2. Simultaneous reduction of activator and repressor functions rescues developmental and circadian period and phase phenotypes. (*A*) Petiole lengths of plants grown in short days (8 h light:16 h dark; *n* = 12–14). (*B*) Hypocotyl lengths of seedlings grown in continuous monochromatic red light (25 μmol m^{-2} s^{-1}; *n* = 24–30). (*C*) Number of leaves produced before bolting in plants grown in LDs (16 h light:8 h dark; *n* = 26–27). (*D*) Free-running period of *CCR2::LUC+* activity (plants maintained in constant 35 μmol m^{-2} s^{-1} red + 35 μmol m^{-2} s^{-1} blue light; *n* = 24–36). Groups that do not share a letter have significantly different mean values ($P < 0.01$, one-way ANOVA with Tukey's post hoc test). (*E*–*H*) Normalized *CCR2::LUC+* activity of plants maintained in (*E*–*G*) LDs or (*H*) continuous light. Col, the wild-type genetic background for all mutants (Col-0). Error bars ± SEM; note that error bars are smaller than symbols in *E*–*H*.

longer hypocotyls (27), hypocotyl length in the *quint* mutant is not distinguishable from the wild type (Fig. 2*B*).

We next examined the timing of the transition from vegetative to reproductive growth. As previously reported, we found a slight delay in *rve468* (27) and advance in *cca1lhy* plants (15) grown in LD, whether measured as number of leaves or as number of days to flowering (Fig. 2*C* and *SI Appendix*, Fig. S2*B*). As seen for vegetative growth phenotypes, the flowering time phenotype of *quint* mutants is more similar to the wild type than to either mutant parent. Thus, simultaneous reduction of both *LHY*-clade and *RVE8*-clade gene function rescues multiple clock-associated developmental phenotypes.

Removal of both Repressors and Activators Rescues Circadian Phase and Period but Not Amplitude. We next examined activity of the circadian-regulated *CCR2::LUC+* reporter in these lines. As expected given previously reported period phenotypes of *rve468* and *cca1lhy* mutants (15, 18, 28), these lines exhibit late- and early-phase phenotypes, respectively, both in LD and short day (Fig. 2 *E* and *F* and *SI Appendix*, Fig. S2 *C*, *D*, *F*, and *G*). In contrast, the phase of the quintuple mutant is indistinguishable from the wild type in LD and closer to it than either parental line in short day (Fig. 2*G* and *SI Appendix*, Fig. S2 *E*–*G*). This rescue of diel phase in the *quint* mutant is consistent with the predictions of our in silico analysis (Fig. 1*F*) and supports the hypothesis that LHY- and RVE8-like proteins antagonistically regulate common clock genes. Furthermore, it suggests that the period phenotype of the *quint* mutant is closer to the wild type than either parental line. Indeed, we found that the free-running period of luciferase activity is intermediate between the short period of *cca1lhy* mutants and the long period of *rve468* mutants (Fig. 2 *D* and *H*). As also predicted by our in silico analysis, rhythmic amplitude of *CCR2::LUC+* activity in plants deficient for both the activator and repressor Mybs is reduced relative to the wild type (Fig. 2*H* and *SI Appendix*, Fig. S2*H*).

We next used qRT-PCR to examine patterns of core clock gene expression in constant light and standard growth conditions. As expected based on our luciferase data, we found that all transcripts show an earlier peak in the *cca1lhy* mutant and a later peak in the *rve468* mutant but that peak phase in the *quint* is close to the wild type (Fig. 3). This same pattern was seen both for EE-containing genes thought to be directly regulated by CCA1 and RVE8 [*TOC1*, *LUX*, *ELF4*, *GI*, *PRR5*, and *PRR9* (12)] and for genes that do not have EE in their promoters (*ELF3* and *PRR7*) that may be indirectly regulated by these antagonistic Mybs.

Although near–wild-type phases of clock gene expression are observed in the *quint* line, rhythmic amplitude is reduced for most transcripts (Fig. 3). While peak transcript levels of a few genes (*ELF3* and *LUX*) are not reduced relative to the wild type, peak levels of most transcripts, especially *PRR5*, *PRR7*, and *PRR9*, are lower. In addition, trough levels of most transcripts, most notably *ELF3*, are elevated in the *quint* compared with the wild type. Together, these changes in waveform result in statistically significantly lower rhythmic amplitude for almost all of these clock-regulated genes in the *quint* mutant compared with the wild type (*SI Appendix*, Fig. S3). This reduction in amplitude in the *quint* is similar to that observed for *CCR2::LUC+* luciferase activity (Fig. 2*H* and *SI Appendix*, Fig. S2*H*) and is consistent with the predictions of our in silico model (Fig. 1*F*).

The Low-Amplitude *quint* Oscillator Is Sensitized to Genetic and Environmental Perturbation. We next examined the robustness of circadian rhythms in the *quint* background to various perturbations. Overexpressing RVE8 at high levels using the strong *35S* promoter has previously been reported to cause a short-period phenotype (17). We examined the effects of driving more moderate overexpression of *RVE8* using the *UBQ10* promoter (*SI Appendix*, Fig. S4 *A* and *B*). In wild-type T1 plants, the largest

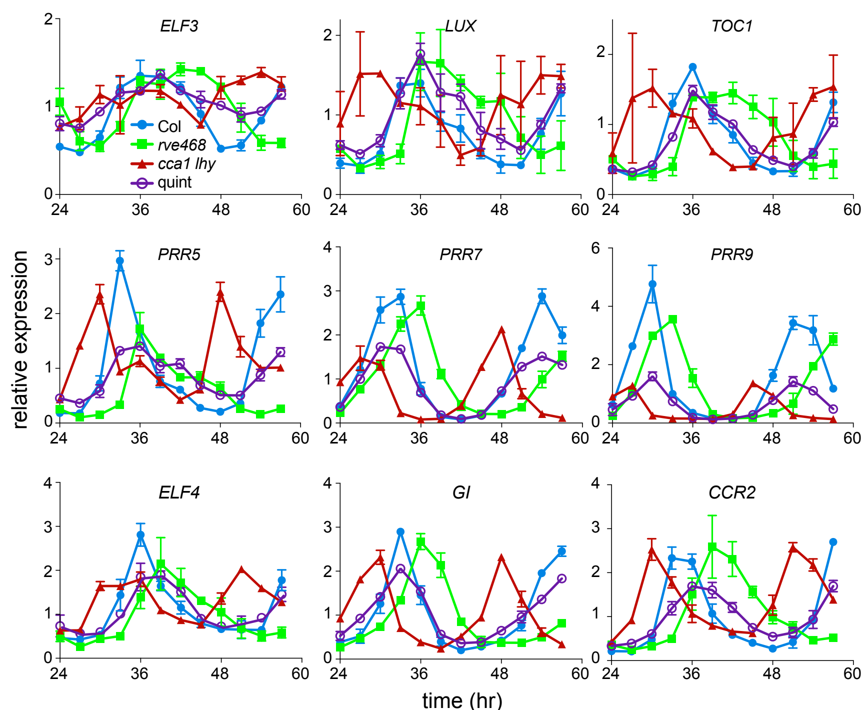

Fig. 3. Loss of both repressors and activators rescues circadian-phase phenotypes but reduces cyclic amplitude of most clock genes. Expression of indicated genes was determined relative to the reference gene *PP2A* by qRT-PCR. Plants were harvested at the indicated times after release into continuous 17.5 μmol m^{-2} s^{-1} red + 17.5 μmol m^{-2} s^{-1} blue light at 22 °C. Col, the wild-type genetic background for all mutants (Col-0). Error bars ± SEM for two biological replicates.

effect of expression of this transgene was a 2-h decrease in free-running period (Fig. 4*A*). In contrast, circadian period in the *quint* T1 plants was 4–6 h shorter than nontransformed controls, and rhythmic amplitude was reduced relative to the wild-type T1 plants (*SI Appendix*, Fig. S4*C*). This difference in phenotype between the wild type and the *quint* could not be attributed to different levels of RVE8 expression (Fig. 4*A* and *SI Appendix*, Fig. S4 *A* and *B*). Thus, the circadian clock of *quint* plants is more susceptible to genetic perturbation than that of wild-type plants.

One characteristic of circadian clocks in diurnal organisms, such as plants, is that at high fluence rates the clock runs slightly faster than at low fluence rates (29). To test how the quintuple mutant responds to changes in the light environment, we examined period and rhythmicity across different fluence rates of light (*SI Appendix*, Fig. S5*A*). Period in the *quint* mutant is similar to the wild type at all fluence rates tested; however, the slope of the response is opposite in the two genotypes. We next examined rhythmic robustness as measured as relative amplitude error (RAE). The RAE of a perfect sine wave is defined as 0, while an RAE = 1 indicates no significant rhythm (the error in the amplitude is equal to the amplitude value itself). The RAEs of both *cca1lhy* and *quint* plants are higher than in the wild type at all light intensities, indicating lower rhythmic robustness in these mutants (*SI Appendix*, Fig. S5 *B–I*).

Another defining characteristic of circadian clocks is their ability to maintain a relatively unchanged free-running period across a range of temperatures, a phenomenon termed temperature compensation (30, 31). To test the effect of altered ambient temperatures on clock function, we entrained the control and mutant lines in light/dark cycles at 22 °C and then monitored *CCR2::LUC+* activity after transfer to constant light at 12 °C, 22 °C, or 30 °C, all physiologically relevant temperatures for *Arabidopsis*. As expected, free-running period in the wild type is largely unchanged over this temperature range (Fig. 4*B*). Period in the *rve468* mutants is undercompensated, with increased temperature resulting in a shortening of free-running period.Conversely, period in plants overexpressing *RVE8* is overcompensated, with free-running period lengthening with increases in temperature. Although *cca1lhy* mutants are arrhythmic at 12 °C, as previously reported (7), their longer period at 30 °C than at 22 °C also shows overcompensation. The *quint* mutant has a slightly shorter period at 30 °C than at lower temperatures and thus, is slightly undercompensated relative to the wild type, although less so than *rve468* plants. Together, these data indicate that an appropriate balance of expression between the activator- and repressor-type Mybs is required for temperature compensation of free-running period.

In addition to period stability at different temperatures, another important trait is rhythmic robustness across different environmental conditions. Wild-type plants have robust rhythms at all temperatures tested: 12 °C, 22 °C, and 30 °C (Fig. 4*C* and *SI Appendix*, Fig. S6). In contrast, at 12 °C, *CCR2::LUC+* activity in both *cca1lhy* and *RVE8* overexpressing plants is almost completely arrhythmic: one-half of the plants did not return any period estimate, and the ones that did had very high RAE values (*SI Appendix*, Fig. S6*D*). The *quint* mutant is less rhythmic than the wild type at 12 °C but much more so than *cca1lhy*, with only 3% of *quint* plants failing to return a period estimate. Of those *quint* that did return a period estimate, the median RAE was intermediate between that of the wild type and *cca1lhy*. The partial rescue of rhythmicity in *quint* relative to *cca1lhy* mutants and the arrhythmicity of *35S::RVE8* plants indicate that a balance between LHY-type and RVE8-type Mybs is essential for rhythmicity at low temperatures. Similarly, while luciferase rhythms in the wild type are robust at 30 °C, *cca1lhy* and *quint* mutants both have very poor rhythms with high and indistinguishable RAE values. In contrast, *35S::RVE8* plants have RAE values close to the wild type (Fig. 4*C* and *SI Appendix*, Fig. S6). These data suggest that LHY clade function is indispensable for rhythmicity at high temperatures but that balanced expression between activator- and repressor-type Mybs is not an important factor. Together, these data show that the Mybs play a key role in the maintenance of cyclic robustness at nonoptimal temperature conditions but are not essential under favorable conditions.

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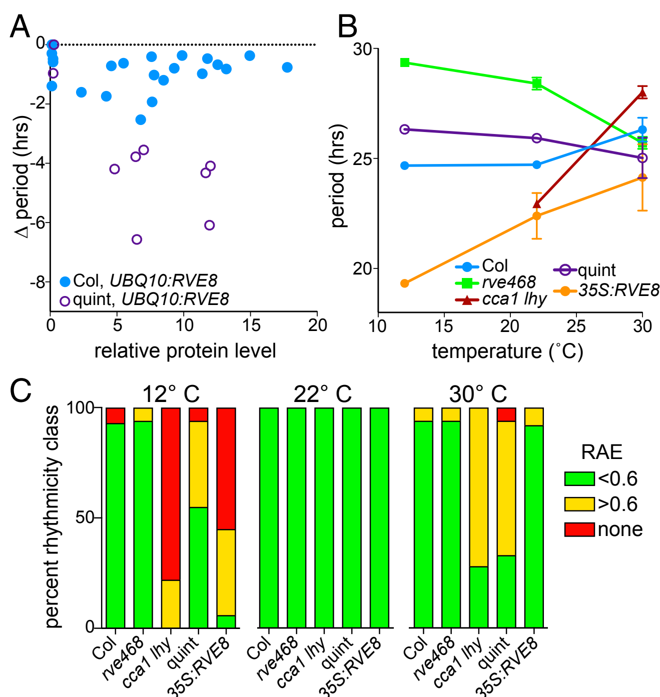

Fig. 4. Loss of both repressors and activators reduces clock robustness. (*A*) RVE8 protein levels (normalized to actin) in T1 *UBQ10p::RVE8-Ypet-3xFLAG* plants (Col and *quint* genetic backgrounds) are plotted vs. the difference in period between each T1 plant and its background genotype. (*B*) Period estimates of *CCR2::LUC+* activity in plants grown at 22 °C in light/dark cycles and then released into constant 35 μmol m^{-2} s^{-1} red + 35 μmol m^{-2} s^{-1} blue light at the indicated temperatures. Mean periods are only plotted for samples with RAE < 0.6 (*n* = 29–36, 11–18, and 5–54 for 12 °C, 22 °C, and 30 °C, respectively, except that, at 12 °C, *n* = 1 for *35S:RVE8*). Error bars ± SEM. (*C*) Rhythmic amplitude (measured as RAE) at different temperatures. Plants were grown as in *B*. Col, the wild-type genetic background for all mutants (Col-0).

Repressor- and Activator-Type Mybs Are Deeply Conserved. The finding that expression of both activator- and repressor-type Mybs is important for both rhythmic amplitude at favorable temperatures and rhythmic robustness under nonoptimal conditions prompted us to investigate whether these distinct factors are of ancient origin or a recent evolutionary innovation. A phylogenetic tree of homologous proteins encoded by angiosperm, nonvascular land plants, and green algae genomes revealed clades containing LHY-like and RVE8-like proteins in these highly diverse species (*SI Appendix*, Fig. S7). The presence of both types of proteins even in unicellular green algae indicates their ancient origin and suggests that these antagonistic transcription factors may have been part of the earliest plant clocks.

Discussion

LHY and RVE8 Clade Proteins Act Antagonistically Within the Plant Oscillator Network. Previous genome-wide studies have revealed that many clock-controlled genes are regulated by both CCA1 and RVE8, with CCA1 primarily acting as a repressor and RVE8 primarily acting as an activator of gene expression (18, 23, 24). To further test this hypothesis, we modified a simple in silico model predicting the effects of coordinate regulation by a cycling activator and a cycling repressor on a clock-regulated gene (19). We found that simulation of reduction of function of the cycling activator or repressor recapitulated the previously reported mutant phenotypes of *cca1lhy* and *rve* mutant plants, respectively (14–18). Simulation of simultaneous reduction of function of both the cycling activator and repressor largely rescued circadian phase but at the expense of reduced circadian amplitude (Fig. 1 *C–F*). Remarkably, the *cca1 lhy rve468* quintuple mutant evinced the near–wild-type period and reduced amplitude phenotypes predicted by our simple model (Figs. 2 *D*, *G*, and *H* and 3 and *SI Appendix*, Figs. S2 *E–H* and S3). This agreement between the model and our observations strongly suggests that the LHY- and RVE8-clade proteins predominantly function antagonistically within the clock network. This is in accord with recent publications reporting that CCA1 and LHY repress expression of even the morning-phased clock genes *PRR7* and *PRR9* (24, 32) instead of acting as activators of these genes as originally suggested (33).

In addition to both binding the same *cis*-element, RVE8- and LHY-clade proteins have both been previously reported to bind to LNK proteins (34). LNK1 and LNK2 act as coactivators with RVE8 to promote expression of clock genes, such as *PRR5*, *TOC1*, and *ELF4*, with RVE8 transactivation strongly attenuated in *lnk1 lnk2* mutants (34, 35). Although the physiological significance of LNK protein binding to CCA1 and LHY for clock function is not clear, it has been reported that LNK1 and LNK2 can act with a distinct type of Myb protein, MYB3, as corepressors of gene expression (36). Thus, it is possible that LNKs may also act as corepressors with LHY-type Mybs. Whether or not LNKs directly facilitate LHY-mediated repression of gene expression, the shared ability of both RVE8- and LHY-clade proteins to bind to LNKs suggests that the antagonism between these types of factors may involve not only competition for common *cis*-elements but also, binding to LNK coregulatory proteins.

Rhythmic gene expression in *quint* mutants in diel conditions is characterized both by reduced amplitude and by additional subtle alterations in waveform. In both *cca1lhy* and *quint* mutants maintained in either short day or LD, there is a transient peak in *CCR2::LUC+* activity soon after lights on (Fig. 2 *F* and *G* and *SI Appendix*, Fig. S2 *D* and *E*). This may be due to enhanced expression of activators normally repressed by CCA1 or LHY in the morning. Possible candidates include the *RVE8* homologs *RVE3* and *RVE5*, which when mutated, slightly enhance the long-period phenotype of *rve468* plants (27).

Many Clock-Related Developmental Phenotypes Are Due to Differences in Circadian Phase. Disruption of clock function is associated with a number of developmental phenotypes, some of the best studied being alterations in the elongation of organs, such as hypocotyls and petioles, and the photoperiodic regulation of flowering (37). In some cases, these developmental phenotypes are clearly due to changes in circadian phase in the mutants relative to the timing of light/dark transitions (38–40). However, in other cases, mutant phenotypes are also observed in constant environmental conditions, indicating that differences in gene expression levels rather than the phase of clock output genes are responsible for the observed phenotypes (26, 27). Our finding that the growth phenotypes observed in *cca1lhy* and *rve468* plants are largely rescued in *quint* mutants grown either in light/dark cycles or in constant light (Fig. 2 and *SI Appendix*, Fig. S2) suggests that compensatory reduction of the activator- and repressor-type Mybs rescues both the diel phase and expression-level phenotypes of clock output genes that are important for these processes. Furthermore, these data suggest that the balance between expression levels of these antagonistic Mybs is more important for normal development than the absolute levels of either type of factor.

The Multiple Feedback Loops of the Plant Clock Enhance Rhythmicity in Adverse Environmental Conditions. The plant circadian oscillator consists of at least 20 transcriptional regulators connected by an even larger number of feedback loops (11, 12). There is no obvious reason for this surprising degree of complexity: in silico modeling of gene regulatory networks has found that the appropriate phasing of clock outputs can be achieved using only a

few regulatory factors connected by a handful of feedback loops, even in the face of changing photoperiods and noise in the timing of light signals (41). Indeed, circadian clocks in animals are composed of a small number of transcription factors connected by a few feedback loops (13). Thus, circadian clock function per se can be achieved by a limited number of components.

One apparent redundancy in the plant clock network is the use of cycling repressors and activators of gene expression to control expression of common target genes, since the activity of one or the other type of regulatory factor would suffice to generate rhythmic outputs (19). To investigate the functional relevance of these factors, we generated plants mutant for the antagonistic *LHY*- and *RVE8*-type Mybs. As predicted by our in silico model (Fig. 1), loss of both types of factors largely restored a wild-type circadian period but reduced rhythmic amplitude even under optimal growth conditions (Fig. 3 and *SI Appendix*, Fig. S3). Despite this reduced amplitude, free-running rhythms in the *quint* mutant are robust under these conditions. However, at physiologically relevant but nonoptimal temperatures, rhythmicity is severely reduced in the *quint* mutant relative to the wild type (Fig. 4 and *SI Appendix*, Fig. S6). Thus, activity of the antagonistic Myb factors is needed to promote rhythmicity under adverse but not optimal growth conditions.

Our observation that both *LHY*- and *RVE8*-type Myb factors can be found in separate clades across the land plant lineage and in some unicellular green algae (*SI Appendix*, Fig. S7) indicates that these distinct types of factors are of ancient evolutionary origin. Thus, they may have functioned in even the earliest arising plant circadian oscillators. As sessile, poikilothermic organisms, plants cannot modify or evade daily and seasonal changes in temperature. It may be that the high degree of complexity of the plant circadian clock, including these antagonistic Myb proteins, is required for robust rhythmicity across the diverse environmental conditions to which plants are regularly subjected.

Materials and Methods

All plants used in this study are in the wild-type Columbia (Col-0) background. Detailed descriptions of the genotypes and growth conditions are described in *SI Appendix, SI Materials and Methods*. Materials, such as plasmids; methods used for phenotypic analysis (luciferase imagining, growth and flowering time assays, Western blotting); and details on the mathematical modeling and statistical analyses are also reported in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Julin Maloof for assistance with statistical analyses and members of the laboratory of S.L.H. for helpful discussions. We also thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants (42) and the Arabidopsis Biological Resource Center (https://abrc.osu.edu/) for seed distribution. This work was supported by National Institutes of Health Grant R01 GM069418 (to S.L.H.) and US Department of Agriculture–National Institute of Food and Agriculture Grant CA-D-PLB-2259-H (to S.L.H.).

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