

LNK1 and LNK2 Are Transcriptional Coactivators in the *Arabidopsis* Circadian Oscillator^{WJ|OPEN}

Qiguang Xie,^{a,1} Peng Wang,^{a,1} Xian Liu,^{a,1} Li Yuan,^a Lingbao Wang,^a Chenguang Zhang,^a Yue Li,^a Hongya Xing,^a Liya Zhi,^a Zhiliang Yue,^a Chunsheng Zhao,^a C. Robertson McClung,^b and Xiaodong Xu^{a,2}

^aHebei Key Laboratory of Molecular and Cellular Biology, Key Laboratory of Molecular and Cellular Biology of the Ministry of Education, College of Life Sciences, Hebei Normal University, Hebei Collaboration Innovation Center for Cell Signaling, Shijiazhuang, Hebei 050024, China

^bDepartment of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755-3563

ORCID ID: 0000-0002-8795-7651 (X.X.)

Transcriptional feedback loops are central to the architecture of eukaryotic circadian clocks. Models of the *Arabidopsis thaliana* circadian clock have emphasized transcriptional repressors, but recently, Myb-like REVEILLE (RVE) transcription factors have been established as transcriptional activators of central clock components, including PSEUDO-RESPONSE REGULATOR5 (PRR5) and TIMING OF CAB EXPRESSION1 (TOC1). We show here that NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED1 (LNK1) and LNK2, members of a small family of four LNK proteins, dynamically interact with morning-expressed oscillator components, including RVE4 and RVE8. Mutational disruption of LNK1 and LNK2 function prevents transcriptional activation of PRR5 by RVE8. The LNKs lack known DNA binding domains, yet LNK1 acts as a transcriptional activator in yeast and in planta. Chromatin immunoprecipitation shows that LNK1 is recruited to the PRR5 and TOC1 promoters in planta. We conclude that LNK1 is a transcriptional coactivator necessary for expression of the clock genes PRR5 and TOC1 through recruitment to their promoters via interaction with bona fide DNA binding proteins such as RVE4 and RVE8.

INTRODUCTION

Circadian clocks enable organisms to coordinate with diurnal changes in the environment; thus, clock function enhances and clock dysfunction diminishes fitness (Dodd et al., 2005; Graf and Smith, 2011). The plant circadian clock is composed of interlocked transcriptional feedback loops that include both activating and repressive components (Hsu and Harmer, 2014; McClung, 2014). The first loop to be described entails the reciprocal repression of the evening-expressed gene *TIMING OF CAB EXPRESSION1* (*TOC1*) by two morning-expressed genes, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*). The transcriptional repression of *TOC1* by *CCA1* and *LHY* requires the recruitment of the transcriptional corepressor *DEETIOLATED1* (*DET1*), likely as part of a larger *COP10-DET1-DDB1* complex (Lau et al., 2011). *TOC1* (also called *PRR1*) is the founding member of a small *PSEUDO-RESPONSE REGULATOR* (*PRR*) family. *TOC1* is a transcriptional repressor of multiple genes, including not only *CCA1* and *LHY* but also *PRR9*, *PRR7*, *PRR5*, and the evening-expressed genes *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING4* (*ELF4*), and *GIGANTEA* (*GI*) (Gendron et al., 2012; Huang et al., 2012;

Pokhilko et al., 2012). *TOC1* represses *CCA1* and *LHY* from its induction at dusk until slightly before dawn; the sequential expression of *PRR9*, *PRR7*, and *PRR5* broadens the temporal domain of *CCA1* and *LHY* repression to begin shortly after dawn and to continue through the induction of *TOC1* at dusk and extend until shortly before dawn (Nakamichi et al., 2010). Thus, *CCA1* and *LHY* transcription is limited to a narrow window around dawn. *PRR9*, *PRR7*, and *PRR5* bind to the *CCA1* and *LHY* promoters and recruit transcriptional corepressors of the Groucho/Tup1 corepressor family, *TOPLESS/TOPLESS-RELATED* (*TPL/TPR*), to repress *CCA1* and *LHY* transcription (Wang et al., 2013). The mechanism by which *TOC1* represses *CCA1* and *LHY* transcription is less completely understood; *TOC1* may possess intrinsic repressor activity, but *CCA1* *HIKING EXPEDITION* (*CHE*) interacts with *TOC1* and contributes to this repression (Prunedapaz et al., 2009).

CCA1 and *LHY* are members of a small gene family, including *REVEILLE* (*RVE*) genes, that encodes Myb domain transcription factors (Carré and Kim, 2002). Although *CCA1* and *LHY* are best defined as transcriptional repressors, genetic experiments show them to positively regulate the expression of *PRR9* and *PRR7* (Farré et al., 2005), and chromatin immunoprecipitation shows that *CCA1* and *LHY* bind to the *PRR9* and *PRR7* promoters (Portolés and Más, 2010). Some *RVE* genes, including *RVE4*, *RVE6*, and *RVE8* (also called *LHY-CCA1-LIKE5*), have been placed in positive regulatory roles in clock feedback loops (Hsu and Harmer, 2014). For example, *RVE8* promotes the expression of *PRR5* and *TOC1* as well as other evening genes, including *LUX*, *ELF4*, and *GI*, by directly binding to the evening element (EE) of their promoters (Farinas and Mas, 2011; Rawat et al., 2011; Hsu et al., 2013).

¹ These authors contributed equally to this work.

² Address correspondence to xiaodong.xu@hebtu.edu.cn.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Xiaodong Xu (xiaodong.xu@hebtu.edu.cn).

WJ| Online version contains Web-only data.

OPEN| Articles can be viewed online without a subscription.

www.plantcell.org/cgi/doi/10.1105/tpc.114.126573

LNK1 and *LNK2*, two members of a family of *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED* genes, were recently demonstrated to be important for wild-type period determination (Rugnone et al., 2013). Here, we report that *LNK1* and *LNK2* physically interact in the nucleus with multiple dawn-phased transcription factors, including *CCA1*, *LHY*, *RVE4*, and *RVE8*. *LNK1* and *LNK2* have not been shown to bind directly to DNA, yet they possess transcriptional activator activity. Expression of *PRR5* and *TOC1* is perturbed in mutants lacking *LNK1* and *LNK2* function. We show that *LNK1* is recruited to fragments of the *PRR5* and *TOC1* promoters that contain EEs, the binding targets of *CCA1*, *LHY*, *RVE4*, and *RVE8*. We propose that *LNK1* and *LNK2* are transcriptional coactivators required for the activation of *PRR5* and *TOC1* transcription by *RVE8* and possibly by *RVE4* and other transcription factors.

RESULTS

Mutation of *LNK1* and *LNK2* Disturbs Multiple Circadian Outputs

To identify components contributing to the *Arabidopsis thaliana* circadian transcriptional network, we examined the genome-wide expression profile of NASCArrays (Nottingham Arabidopsis Stock Centre Transcriptomics Service) for genes whose expression was highly correlated with the well-known morning-expressed clock genes *CCA1* and *LHY* (Figure 1A). We identified a small gene family, *LNK1* to *LNK4*, recently shown to be important for circadian clock function (Rugnone et al., 2013). *LNK1* to *LNK4* expression oscillates in both shoots and roots, and the *LNK* genes are among a small set of genes whose mRNA abundance continues to oscillate in roots under continuous darkness (James et al., 2008). To confirm that these *LNK* genes are important for circadian clock function, we identified lines homozygous for T-DNA insertions into each *LNK* gene (*lnk1-1*, *lnk2-4*, *lnk3-1*, and *lnk4-1*), introduced a circadian-expressed reporter construct in which firefly *LUCIFERASE* (*LUC*) expression is driven by the promoter of the *LIGHT-HARVESTING CHLOROPHYLL a/b BINDING PROTEIN* gene (*LHCB1*1*; also called *CAB2*), and measured circadian function in seedlings entrained in 12-h-light/12-h-dark cycles before release into free-running conditions of continuous white light and constant temperature. Loss of either *LNK1* or *LNK2* function lengthened the period ~2 h relative to wild-type Columbia-0 (Col-0) (Figure 1B; Supplemental Table 1), while loss of either *LNK3* or *LNK4* function conferred no obvious clock defect (Figure 1C; Supplemental Table 1).

Mutation of *LNK1* or *LNK2* also lengthened the period of clock gene expression (*CCA1:LUC*, *LHY:LUC*, and *TOC1:LUC*) in continuous light (Figure 1D; Supplemental Figure 1 and Supplemental Table 1). The long period phenotypes of *lnk1* and *lnk2* mutants were completely rescued by transgenic complementation with *LNK1* and *LNK2*, respectively, driven by their endogenous promoters (Supplemental Figure 1A and Supplemental Table 1). *LNK1* and *LNK2* are at least partially redundant, because the *lnk1 lnk2* double mutant had a longer period of *CCA1:LUC* expression than either single mutant (Figure 1D) (Rugnone et al., 2013). These observations were confirmed by quantitative RT-PCR (qRT-PCR) assay for the level of steady state mRNA abundance of *CCA1*,

LHY, and *TOC1* and extended to show period lengthening of the expression of *PRR5*, *PRR7*, and *PRR9* in *lnk* single and double mutants (Figure 2). However, overexpression of either *LNK1* or *LNK2* (35S:*LNK1* or 35S:*LNK2*) had no effect on circadian period (Figure 1E; Supplemental Figure 2A and Supplemental Table 1). Thus, although *LNK1* and *LNK2* are necessary for multiple clock-controlled output rhythms, overexpression of either alone is insufficient to disrupt clock function.

LNK1 and *LNK2* Are Clock Regulated and Contribute to Red Light Signaling to the Clock

LNK1 and *LNK2*, like many genes associated with the circadian clock, show robust oscillations in transcription and protein accumulation in both diurnal and free-running conditions. qRT-PCR analysis demonstrated cycling abundance of *LNK1* and *LNK2* steady state mRNA (Rugnone et al., 2013). *LNK* transcriptional (*LNK1:LUC* and *LNK2:LUC*) and translational (*LNK1:LNK-LUC* and *LNK2:LNK-LUC*) fusions show ~24-h rhythms, with peaks occurring in early morning (1.5 to 2 h after subjective dawn) and with the peaks of fusion protein accumulation lagging slightly behind peaks in transcriptional activity (Figure 1F).

Plant circadian clock function is typically sensitive to light, and period typically shortens with increasing light intensity (Salomé and McClung, 2005a). We found that *lnk1* and *lnk2* mutants were insensitive to red light in that period failed to shorten with increasing intensity (Figure 1G; Supplemental Table 2). However, *lnk1* and *lnk2* mutants retained sensitivity to blue light, with period inversely proportional to the light intensity (Figure 1H; Supplemental Table 2). Thus, we conclude that *LNK1* and *LNK2* are directly or indirectly the targets of red but not blue light signaling. In contrast to its sensitivity to light, circadian period typically is relatively insensitive to temperature and remains relatively constant across a range of physiologically relevant temperatures (temperature compensation) (Salomé and McClung, 2005a). *lnk1* and *lnk2* mutants retained wild-type temperature compensation between 12 and 27°C (Figure 1I; Supplemental Table 3).

LNK1 and *LNK2* Are Nuclear Localized and Physically Interact with *CCA1*, *LHY*, *RVE4*, and *RVE8*

LNK1 and *LNK2* expression is widespread in most tissues and organs at all developmental stages tested, as measured in *Arabidopsis* transgenic plants expressing β -glucuronidase (GUS) protein fusions driven by the *LNK* promoters and by qRT-PCR (Supplemental Figure 3). GUS staining was more intense in the vasculature of roots, hypocotyl, and cotyledons and was particularly intense at root tips and in young true leaves. By qRT-PCR analysis, *LNK1* and *LNK2* mRNAs were found to be most abundant in leaves and least abundant in roots. *LNK* is localized to the nucleus, as determined by transient expression in *Nicotiana benthamiana* leaves or in stable transgenic *Arabidopsis* lines expressing fusions of *LNK1* or *LNK2* to green fluorescent protein (GFP; fused in frame to either the C or N terminus of *LNK*) that were driven by the constitutive 35S promoter (Supplemental Figure 4).

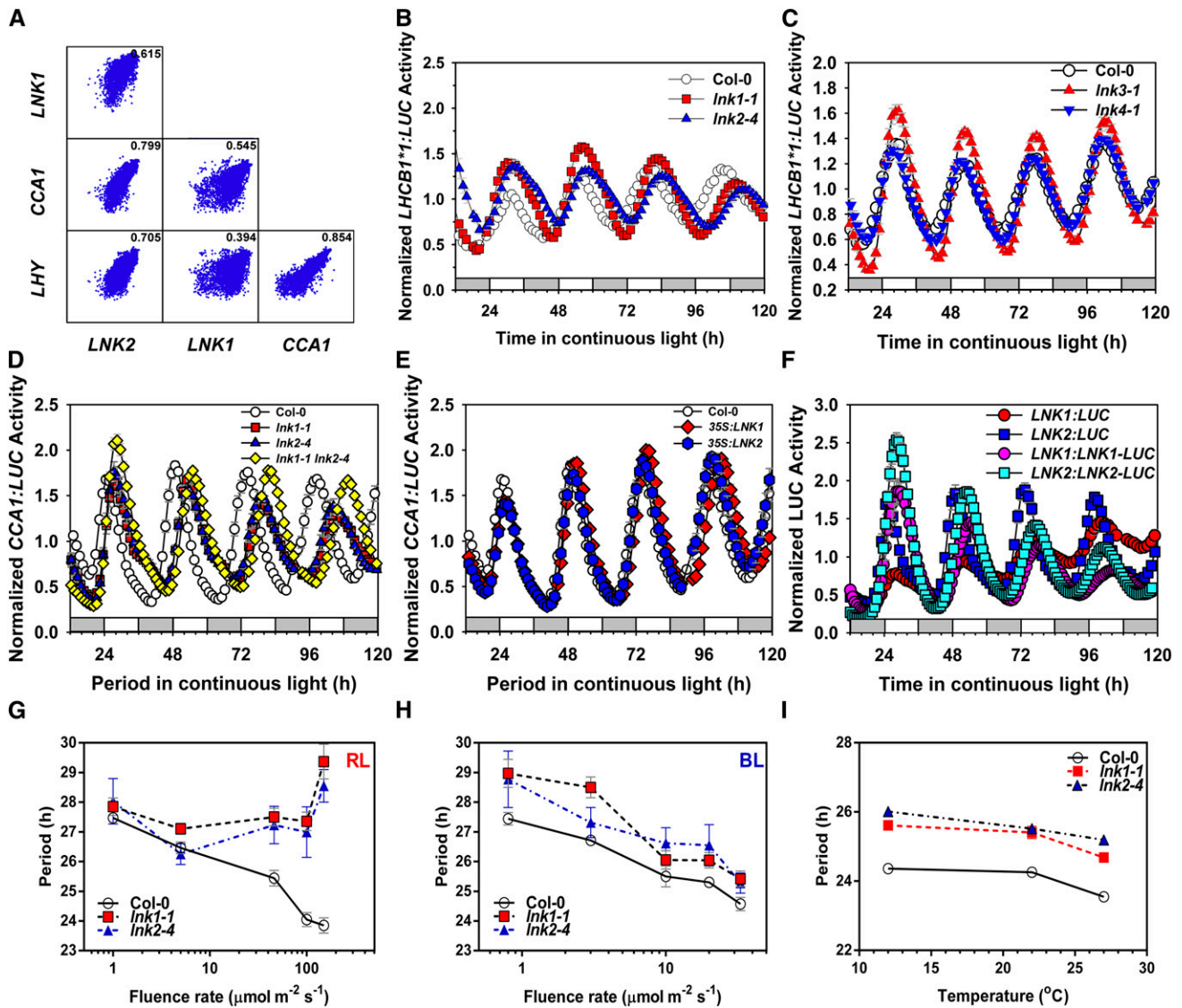


Figure 1. *LNK1* and *LNK2* Are Required to Maintain Circadian Rhythms and Contribute to Red Light Signaling to the Clock.

(A) Correlation of expression of *LNK1*, *LNK2*, *CCA1*, and *LHY* from 5211 individual microarray experiments deposited in the NASCArrays database (<http://affymetrix.arabidopsis.info>). The values in each panel are Pearson correlation coefficients.

(B) to (F) Genetic analysis of the effects of LNK loss of function or overexpression on gene expression as determined with *PROMOTER:LUCIFERASE (PRO:LUC)* gene fusions: *LHCb1*1:LUC* (**(B)** and **(C)**), *CCA1:LUC* (**(D)** and **(E)**), and *LNK:LUC* transcriptional and *LNK:LNK-LUC* translational fusions (**(F)**). The data in **(B)** to **(E)** are summarized in Supplemental Table 1.

(G) to (I) Effects of continuous red **(G)** or blue **(H)** light intensity and of temperature **(I)** on circadian period measured with *CCA1:LUC* in *lnk1-1*, *lnk2-4*, and the wild type (Col-0). The values are means \pm SE from two independent biological replicates. These data are summarized in Supplemental Tables 2 and 3.

LNK1 and *LNK2* show similar circadian, spatial, and developmental expression patterns to *CCA1* and *LHY*, which also localize to the nucleus (Carré and Kim, 2002). Therefore, we asked whether these proteins might be physically associated in plants using bimolecular fluorescence complementation (BiFC) and firefly luciferase complementation imaging (LCI) in *Agrobacterium tumefaciens*-infiltrated *N. benthamiana* leaves. For BiFC, 35S:*LNK1-YFPn* or 35S:*LNK2-YFPn* was coinfiltrated with

35S:*CCA1-YFPc* or 35S:*LHY-YFPc*. For LCI, 35S:*LNK1-nLUC* or 35S:*LNK2-nLUC* was coinfiltrated with 35S:*CCA1-cLUC* or 35S:*LHY-cLUC*. Both *LNK1* and *LNK2* interacted with *CCA1* and *LHY* in both assays with complemented yellow fluorescent protein (YFP) in the nucleus or complemented luciferase activity (Figures 3A and 3B; Supplemental Figure 5). We extended this study to show that the *CCA1/LHY*-related *RVE8* and *RVE4*, which act as positive regulators in the *Arabidopsis* clock (Farinas

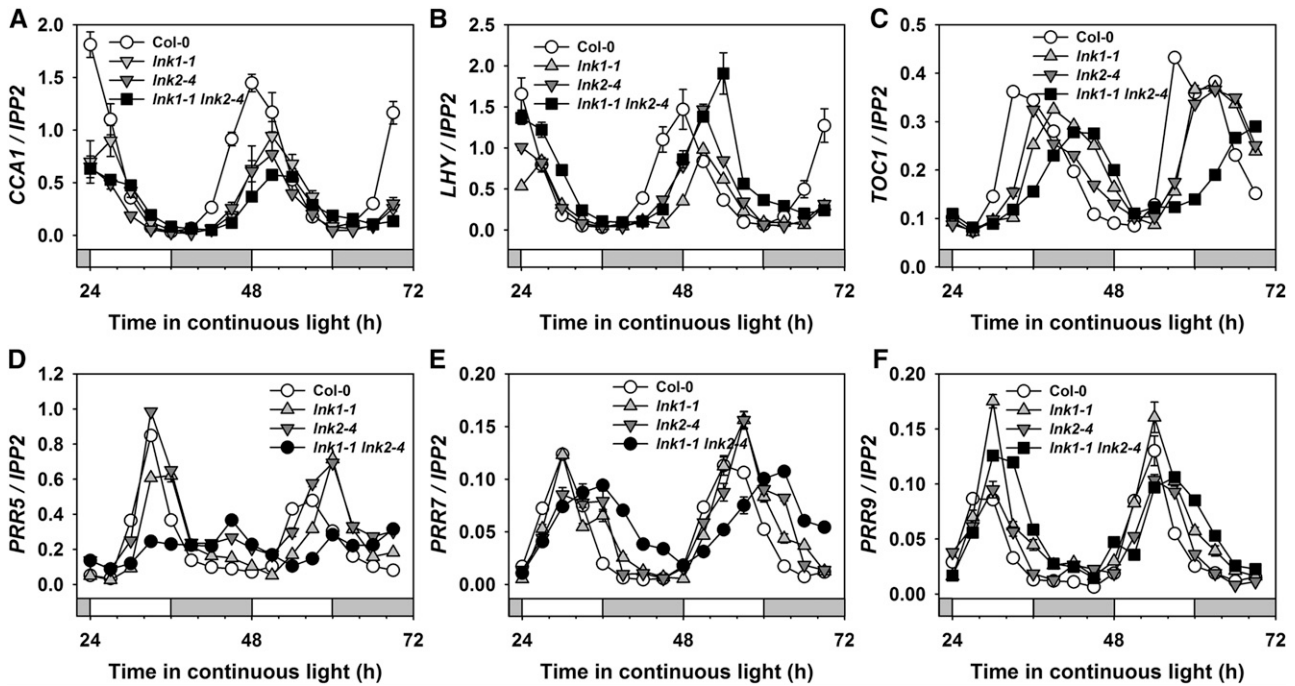


Figure 2. qRT-PCR Analysis of Circadian Clock-Regulated Gene Expression in *Ink1* and *Ink2* Single and *Ink1 Ink2* Double Mutants.

Expression of circadian clock genes in the wild type (Col-0) and in *Ink1-1*, *Ink2-4*, and *Ink1-1 Ink2-4* mutants was compared by qRT-PCR. Seedlings were entrained at 22°C in 12-h-light/12-h-dark photoperiods for 7 d before release to constant light ($70 \mu\text{mol m}^{-2} \text{s}^{-1}$) at ZT0. *IPP2* was used as a noncycling normalization control to quantify the relative expression of *CCA1* (A), *LHY* (B), *TOC1* (C), *PRR5* (D), *PRR7* (E), and *PRR9* (F). The data are presented as means \pm SE of three technical replicates from one of two independent biological experiments; both experiments yielded congruent results.

and Mas, 2011; Rawat et al., 2011; Hsu et al., 2013), both interact with LNK1 and LNK2, as shown with BiFC and firefly LCI in transiently infected *N. benthamiana* leaves (Figures 3C and 3D; Supplemental Figure 5) or in stable *Arabidopsis* transgenic lines (Figures 4A and 4B). We note that these interactions are specific; although LNK1 and LNK2 interact with multiple morning-phased *CCA1/LHY/RVE* proteins, two other morning-phased clock proteins, *PRR9* and *PRR7*, do not interact with LNK1 and LNK2 in yeast two-hybrid assays (Supplemental Figures 6A to 6C). In addition, we note that in yeast two-hybrid assays, both LNK1 and LNK2 fused to an activation domain (AD) interacted with *RVE4* and *RVE8* recruited to DNA via the yeast Gal4 DNA binding domain (BD) (Supplemental Figures 6D and 6E). These interactions could not be confirmed in the reciprocal experiment, because both LNK1-BD and LNK2-BD activated the target in the absence of an interaction partner, indicating that LNK1 and LNK2 each possesses intrinsic transcriptional activation activity (Figure 5A; Supplemental Figures 6D and 6E). Thus, we conclude that LNK1 and LNK2 physically interact in the nucleus with at least four known morning clock transcription factors: *CCA1*, *LHY*, *RVE4*, and *RVE8*.

LNK1 and LNK2 Proteins Interact Dynamically with *CCA1*, *LHY*, *RVE4*, and *RVE8* Proteins in Planta

We confirmed these interactions in stable *Arabidopsis* transgenic lines. We transformed endogenous full-length genomic DNA fusion constructs (*LNK1:LNK1-cLUC*, *LNK2:LNK2-cLUC*,

LNK2:LNK2-nLUC, *CCA1:CCA1-nLUC*, *CCA1:CCA1-cLUC*, *LHY:LHY-nLUC*, *RVE8:RVE8-nLUC*, or *RVE4:RVE4-nLUC*) individually into *Arabidopsis*. Stable transformed lines were crossed to yield F1 seedlings coexpressing pairs of fusions for LUC activity measurement in free-running conditions. All four *LNK-cLUC/RVE-nLUC* combinations showed robust LUC activity oscillations with a period close to 24 h (Figures 4A and 4B). Both *LNK1-cLUC/CCA1-nLUC* and *LNK2-nLUC/CCA1-cLUC* combinations and both *LNK-cLUC/LHY-nLUC* combinations also showed robust oscillations, albeit with a long period (Figures 4A and 4B). For the LNK-*CCA1* interaction, the increased *CCA1* expression associated with the introduction of a second copy of *CCA1* into the wild-type Col-0 background lengthens the period (Supplemental Figure 7), and we conclude that the expression of a second *LHY* copy similarly lengthens the period.

We observed much greater luciferase activity in plants expressing LNK1 or LNK2 with *RVE4* compared with LNK1 or LNK2 with *CCA1*, *LHY*, or *RVE8* (Figures 4A and 4B). This could result from a stronger interaction between LNK1 or LNK2 and *RVE4*. Alternatively, *RVE4* might accumulate to higher levels than *CCA1*, *LHY*, or *RVE8*, which would suggest that Myb abundance is limiting to the interaction. We analyzed microarray data obtained under similar growth conditions (<http://diurnal.mocklerlab.org/>) and found that *RVE4* mRNA accumulated to higher levels than *CCA1*, *LHY*, and *RVE8* mRNAs (Supplemental Figure 8), consistent with this second hypothesis that *RVE4* accumulates to higher levels than *RVE8*, *CCA1*, and *LHY*.

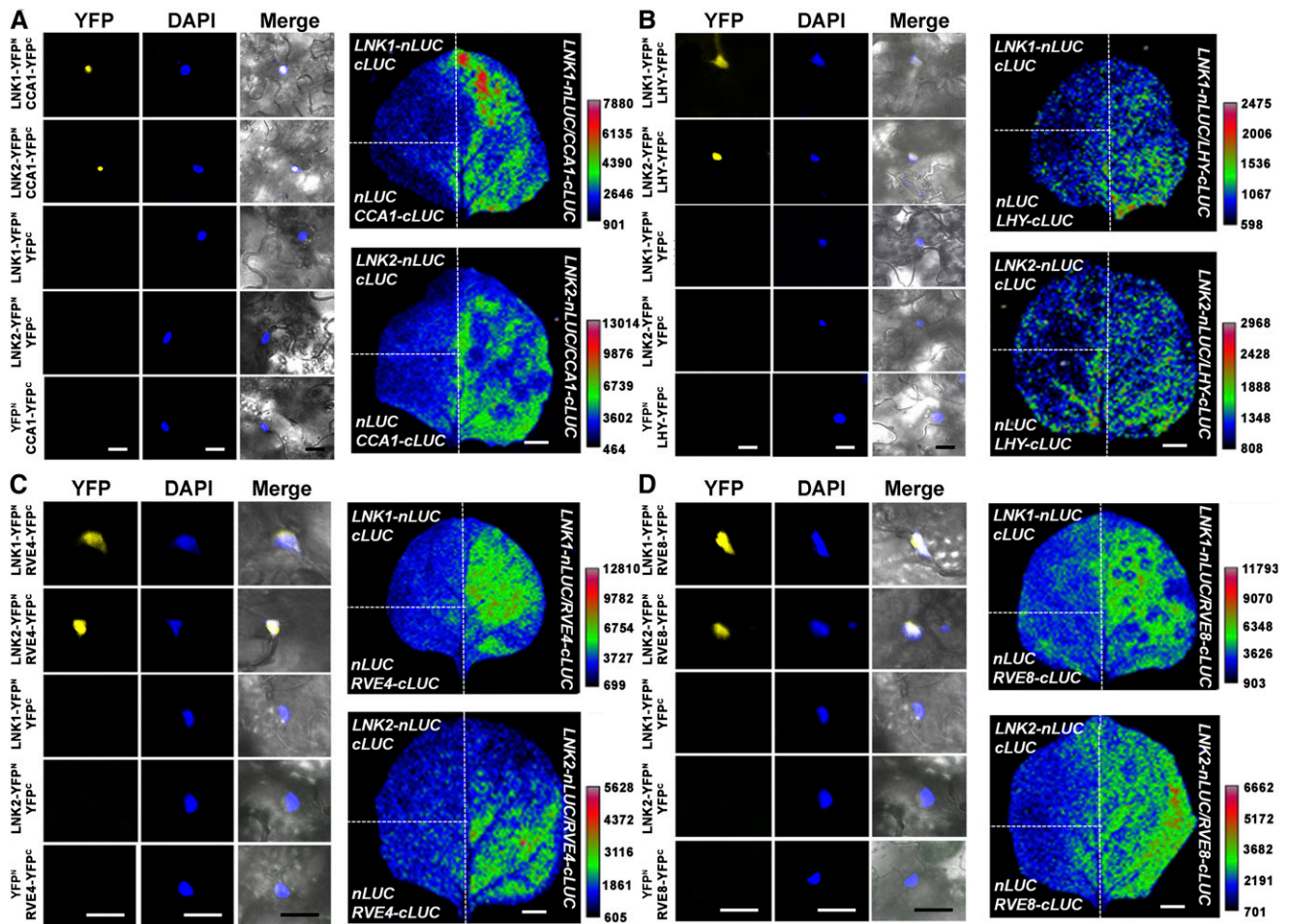


Figure 3. LNK1 and LNK2 Interact with Myb Transcription Factors CCA1, LHY, RVE4, and RVE8 in the Nucleus.

The left three panels show BIFC analysis of LNK1 and LNK2 with CCA1 (A), LHY (B), RVE4 (C), and RVE8 (D) in the nucleus (bars = 20 μm). Fusion constructs in which the N-terminal half of YFP (YFP^N) was fused to LNK1 or LNK2 and the C-terminal half of YFP (YFP^C) was fused to the Myb transcription factor were coinfiltrated into *N. benthamiana* leaves. Confocal microscopy images were captured from epidermal cell layers of transgenic leaves 2 to 3 d after infiltration. Panels on the right show LCI in which both LNK1 and LNK2 interact with CCA1 (A), LHY (B), RVE4 (C), and RVE8 (D) in planta (bars = 1 cm). Overexpression fusion constructs in which the C-terminal half of firefly *LUC* (cLUC) was fused to LNK1 or LNK2 and the N-terminal half (nLUC) was fused to the Myb transcription factor were coinfiltrated into *N. benthamiana* leaves. Images were captured via a low-light charge-coupled device camera.

Two Plant-Specific Conserved Regions Are Important for Dynamic Protein Interactions

Although LNK family members lack known functional domains, *LNK1* to *LNK4* have two highly conserved regions that are plant specific (Figure 4C). Mutation of two residues in either conserved region (Arg-555Asp-556 to GlyGly or Asp-614Arg-615 to GlyGly) of LNK1 was used to test if there are specific regions of LNK1 required for interaction with RVE4. The two mutations significantly reduced LUC complementation of *LNK1:LNK1-cLUC* with *RVE4:RVE4-nLUC* in transgenic *Arabidopsis* seedlings (Figures 4D and 4E). Rhythmic LUC activity was detectable with both mutated LNK1s and RVE4, although the m1-LNK1/RVE4 activity was greater than that observed with m2-LNK1/RVE4. m1-LNK1-cLUC protein abundance was not diminished and may actually be increased relative to wild-type LNK1-cLUC

(Supplemental Figure 9). Thus, the reduction in LUC complementation activity did not result from a loss of m1-LNK1-cLUC protein stability. We conclude that the mutations greatly reduce the ability of LNK1 to interact with RVE4, either by directly disrupting the RVE binding domain or else by generally perturbing the overall protein structure and thereby indirectly compromising the RVE binding domain. Therefore, we conclude that LNK1 and LNK2 physically interact with multiple clock transcription factors (CCA1, LHY, RVE4, and RVE8) at the core of the *Arabidopsis* oscillator through a novel conserved domain found in all four LNK proteins.

Activation of *PRR5* and *TOC1* Transcription by RVE8 Requires LNK1 and LNK2 as Transcriptional Coactivators

If LNK1 or LNK2 physically interacts with multiple clock transcription factors, does either LNK1 or LNK2 possess DNA binding

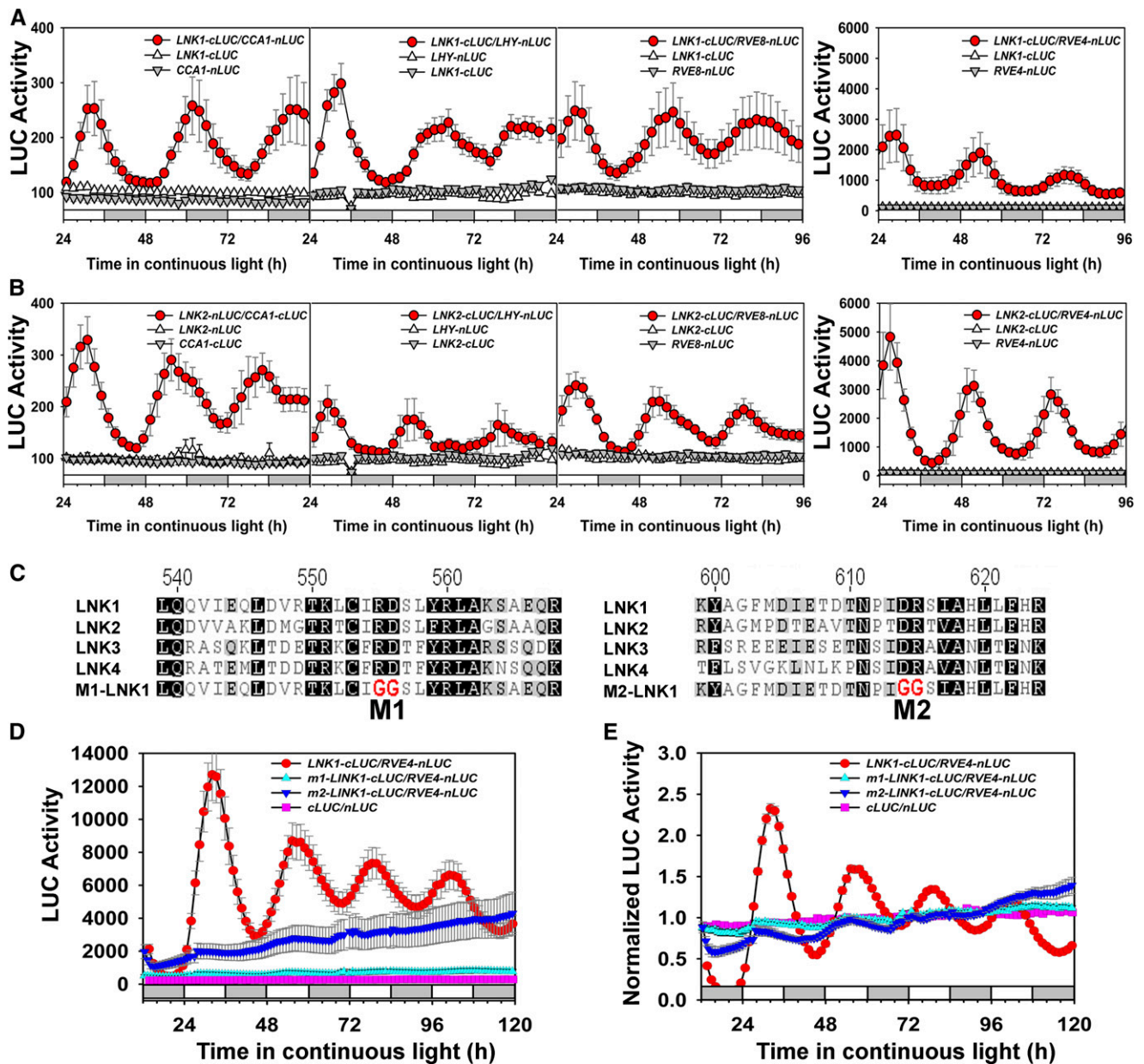


Figure 4. LNK1 and LNK2 Show Dynamic Interactions with Myb Transcription Factors CCA1, LHY, RVE4, and RVE8 in Seedlings, and the Interactions Depend on Two Conserved Domains.

(A) and (B) Endogenous full-length LNK1/LNK2 and CCA1/LHY/RVE4/RVE8 were fused to the N-terminal half of firefly LUC (*nLUC*) or the C-terminal half of firefly LUC (*cLUC*). Constructs were independently transformed into *Arabidopsis* to yield stable transgenic lines that were crossed, and LUC activity was measured in F1 heterozygotes. Data collected with a TopCount luminometer are presented as absolute LUC activities (means \pm SE, $n = 12$ to 24).

(C) Amino acid alignment of LNK1, LNK2, LNK3, and LNK4 reveals two plant-specific conserved domains. Site-specific mutations of the two conserved domains of LNK1 in mutants M1 (RD/GG) and M2 (DR/GG) are indicated in red.

(D) and (E) Luciferase activity (means \pm SE, $n = 24$ to 36) of *Arabidopsis* expressing LNK1:LNK1-cLUC (either the wild type or mutated with M1 or M2) together with RVE4:RVE4-nLUC. Data are presented as absolute expression levels (D) or as normalized data (E), in which the absolute value was divided by the mean value for that trace to allow the visualization of low-amplitude oscillations of *m1-LNK1:LNK1-cLUC* and *m2-LNK1:LNK1-cLUC* with RVE4:RVE4-nLUC.

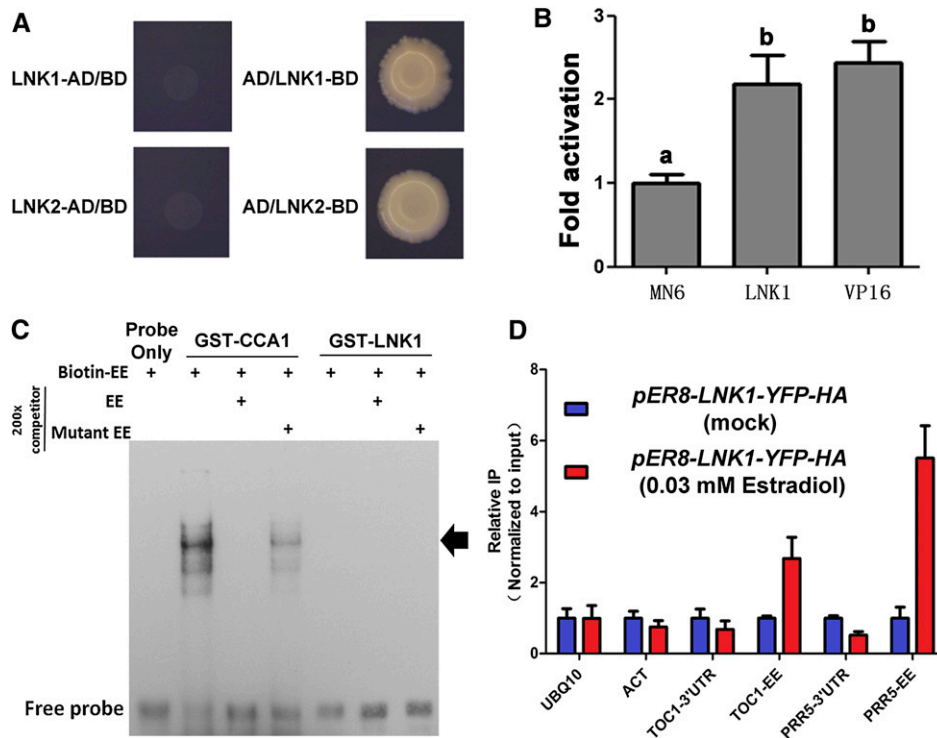


Figure 5. LNK1 and LNK2 Are Transcriptional Activators and Are Recruited to the *TOC1* and *PRR5* Promoters.

(A) LNK1 and LNK2 have transcriptional activity in yeast, as indicated by prototrophy restoration and cell growth of the yeast AH109 strain on selective medium without Leu, Trp, His, and Ade when fused to the Gal4 DNA binding domain (BD) and recruited to the Gal4 DNA binding site.

(B) LNK1 has transcriptional activator activity in planta when transiently expressed in plant mesophyll protoplasts. Transcriptional activity was measured with the use of a dual-luciferase assay system (Promega) in which firefly LUC is driven by the minimal cauliflower mosaic virus 35S promoter Gal4 UAS in the pGLL reporter. Transfection efficiency was normalized by coinfection with 35S-driven Renilla luciferase. Expression driven by LNK1-Gal4-BD (LNK1), presented as means \pm SD ($n \geq 4$), is expressed relative to that of the effector plasmid (pMN6) alone. VP16 has known transcriptional activation activity and serves as a positive control. Different letters indicate significant differences ($P < 0.001$) as determined by ANOVA.

(C) EMSA of a probe including the EE with CCA1-GST and LNK1-GST purified from *E. coli* BL21 (DE3). The arrow indicates the CCA1/EE complex.

(D) Chromatin immunoprecipitation of an estradiol-inducible YFP/HA-tagged LNK1 (*pER8-LNK1-YFP-HA*) with an anti-GFP antibody. Tissue from 2-week-old seedlings treated at ZT0 with DMSO (blue bars) or with 0.03 mM estradiol (red bars) was harvested at ZT28. Immunoprecipitated DNA was quantified by qRT-PCR using *TOC1*- and *PRR5*-specific primers flanking the EE. Primers specific to *ACTIN7* (*ACT*), *UBIQUITIN10* (*UBQ10*), *PRR5* 3' UTR, and the *TOC1* 3' UTR were used as negative controls. The data are presented as means \pm SD of three technical replicates.

[See online article for color version of this figure.]

ability? Each of the LNK interaction partners binds to the EE promoter motif. However, we failed to detect LNK1 binding to the EE via electrophoretic mobility shift assay (EMSA) employing *Escherichia coli*-produced GST-tagged LNK1 (Figure 5C). This, however, would not preclude either LNK1 or LNK2 binding to the EE when complexed to a binding partner or to some other DNA sequence.

As noted above, both LNK1-BD and LNK2-BD activate transcription when recruited to the Gal4 upstream activating sequence (UAS) (Figure 5A; Supplemental Figures 6D and 6E), suggesting that both LNKs possess intrinsic transcriptional activation activity in yeast. Therefore, we investigated whether LNK1 can activate transcription in *Arabidopsis* protoplasts. LNK1 fused to the Gal4-BD and driven by the 35S promoter activated transcription of a reporter construct consisting of a minimal 35S promoter with the Gal4 UAS driving *LUC* as effectively as did the strong VP16 activator domain (Sadowski

et al., 1988) (Figure 5B). Thus, LNK1 functions as a transcriptional activator in planta.

To assess potential regulatory targets of LNK1 and LNK2, we considered the effects of LNK loss of function on the expression of clock genes. mRNA accumulation for *CCA1*, *LHY*, *PRR9*, and *PRR7* showed long period in *lnk* single and double mutants, but the mRNA abundance of *LHY*, *PRR9*, and *PRR7* was largely unperturbed (Figures 2B, 2E, and 2F). By contrast, *PRR5* and, to a lesser extent, *CCA1* and *TOC1* mRNA accumulation was compromised in *lnk* single and double mutants (Figures 2A, 2C, and 2D) (Rugnone et al., 2013), suggesting that LNK1 and LNK2 might be coactivators for *PRR5*, *TOC1*, and *CCA1* transcription. We developed an estradiol-inducible version of LNK1 and showed that induction of LNK1 resulted in increased expression of both *PRR5* and *TOC1* mRNAs (Figure 6). When estradiol was added at Zeitgeber time 0 (ZT0), *LNK1* mRNA was increased relative to mock-treated controls within 3 h, with maximal

induction after 12 h (Figure 6A). Both *PRR5* and *TOC1* mRNAs were increased in induced relative to uninduced seedlings 6 and 12 h after LNK1 induction (Figures 6B and 6C). In both uninduced and induced seedlings, *PRR5* and *TOC1* mRNA abundances were maximal at ZT6 and ZT12, respectively, consistent with their normal circadian phasing. To further confirm the induction of *TOC1* expression by LNK1, we added estradiol at ZT48 (subjective dawn) and observed that induction of LNK1 increased *TOC1* expression (measured as luciferase activity from a *TOC1:LUC* transcriptional fusion) at dusk for each of the next three cycles. When LNK1 was induced by estradiol addition at ZT60 (subjective dusk), *TOC1* expression was not increased until 24 and 48 h later (Figure 6D). We attribute this lag to the induction kinetics of LNK1; LNK1 was detectably increased 12 and 24 h but not 6 h after estradiol addition (Supplemental Figure 2B).

RVE8 has recently been established as an activator of *PRR5* and *TOC1* transcription (Rawat et al., 2011; Hsu et al., 2013). However, we noticed that RVE4 and RVE8, unlike LNK1 and LNK2, failed to activate transcription in yeast when recruited to the reporter gene promoter through fusion to the Gal4 DNA binding domain (Supplemental Figure 6). Therefore, we asked whether LNK1 and LNK2 were coactivators necessary for the induction of *PRR5* and *TOC1* transcription by RVE8. First, we developed an estradiol-inducible version of RVE8 and established that induction increased *PRR5:LUC* expression (Figure 7A). However, this induction was blocked in the *Ink1 Ink2* double mutant background (Figure 7A). Second, *PRR5:LUC* expression is elevated in protoplasts overexpressing RVE8 from the constitutive 35S promoter, but the stimulation of *PRR5:LUC* by RVE8 overexpression was blocked in the *Ink1 Ink2* double mutant (Figure 7B). In *Arabidopsis* transgenic lines carrying an estradiol-inducible RVE8, we observed estradiol-mediated induction of RVE8, *PRR5*, and *TOC1* mRNAs in the wild-type Col-0 background. However, in the *Ink1 Ink2* double mutant, estradiol addition induced RVE8 mRNA, but induction of *PRR5* and *TOC1* was reduced relative to that observed in Col-0 (Figures 7C to 7F).

The data presented above indicate that LNK1 and LNK2 serve as transcriptional coactivators for *PRR5* and *TOC1* transcription, which suggests that LNK1 and LNK2 should be recruited to the *PRR5* and *TOC1* promoters. We used chromatin immunoprecipitation to show that an estradiol-inducible version of LNK1 is recruited to EE-containing fragments of both the *PRR5* and *TOC1* promoters (Figure 5D). Because LNK1 does not bind to the EE in vitro (Figure 5C), we conclude that it is recruited to the *PRR5* and *TOC1* promoters via protein-protein interaction with DNA binding proteins such as RVE8 and RVE4. Are RVE4 and RVE8 necessary for the induction of *PRR5* transcription by LNK1? When we transiently expressed 35S: *LNK1-FLAG* in Col-0 or in *rve8* or *rve4* mutant protoplasts carrying the *PRR5:LUC* reporter, we found that the activation of *PRR5* transcription by LNK1 was significantly reduced in the mutants compared with Col-0 (Figure 6E; Supplemental Figure 2C).

We conclude that LNK1 and LNK2 are coactivators for the induction of *PRR5* transcription that are recruited to the *PRR5* promoter through their interaction with the DNA binding proteins,

RVE8 and RVE4. We note, however, that induction of RVE8 elevated *PRR5* and mRNA abundance even in the *Ink1 Ink2* background, suggesting either that RVE8 has intrinsic transcriptional activation activity or that there are additional coactivators capable of recruitment to the *PRR5* and *TOC1* promoters either via interaction with RVE8 or via an indirect means. For example, RVE8 could induce a second transcriptional activator that is recruited to the *PRR5* and *TOC1* promoters.

DISCUSSION

LNK1 and LNK2 Play Roles in Red Light Input to the Clock

The circadian clock is entrainable by environmental light/dark cycles through signaling pathways associated with multiple photoreceptors. Fluence response curves show that *Ink1* and *Ink2* are impaired in red light signaling to the circadian clock compared with the wild type under constant red light, as period fails to shorten with increasing fluence rate (Figure 1G; Supplemental Table 2). No such defect was detected in blue light signaling, as the period of *Ink1* or *Ink2* mutants shortened in response to increased fluence rate similarly to the wild type; although the period of either *Ink* mutant was longer than that of Col-0, the sensitivity to blue light, as indicated by the slope of the curve, was similar to that of Col-0 (Figure 1H; Supplemental Table 2). In a separate analysis of light sensitivity, *Ink1* and *Ink2* single mutants had longer hypocotyls than wild-type seedlings under constant white and red light conditions and were late flowering under long-day conditions (16 h of light and 8 h of dark) (Rugnone et al., 2013). LNK1 and LNK2 function in red light signaling is at least partially redundant, as the *Ink1 Ink2* double mutant had significantly longer hypocotyls than single *Ink1* and *Ink2* mutants (Rugnone et al., 2013). Collectively, these data establish that LNK1 and LNK2 play a role in red light input to the clock, in photomorphogenesis, and in developmental (flowering) timing.

LNK1 and LNK2 Serve as Transcriptional Coactivators

The plant circadian clock, like those of fungi and animals, includes multiple interlocked transcriptional feedback loops with both activating and repressive components (Dunlap, 1999; Zhang and Kay, 2010). The *Arabidopsis* clock is the best studied among plant clocks, and many clock components have been identified (Hsu and Harmer, 2014; McClung, 2014). Although many clock components encode DNA binding transcription factors, the mechanistic details of how these transcription factors modulate transcription are not fully understood. Among examples of transcriptional repression, multiple diverse mechanisms have been described. For example, CCA1 and LHY repress evening-phased *TOC1* and *GI* transcription via recruitment of the COP10-DET1-DDB1 complex, in which DET1 serves as a transcriptional corepressor (Lau et al., 2011). CCA1 and LHY repress *ELF4* transcription in the morning by binding to and antagonizing three transcriptional activators, FAR-RED ELONGATED HYPOCOTYL3 (FHY3), FAR-RED IMPAIRED RESPONSE1 (FAR1), and LONG HYPOCOTYL5 (HY5) (Li et al., 2011). Transcription of

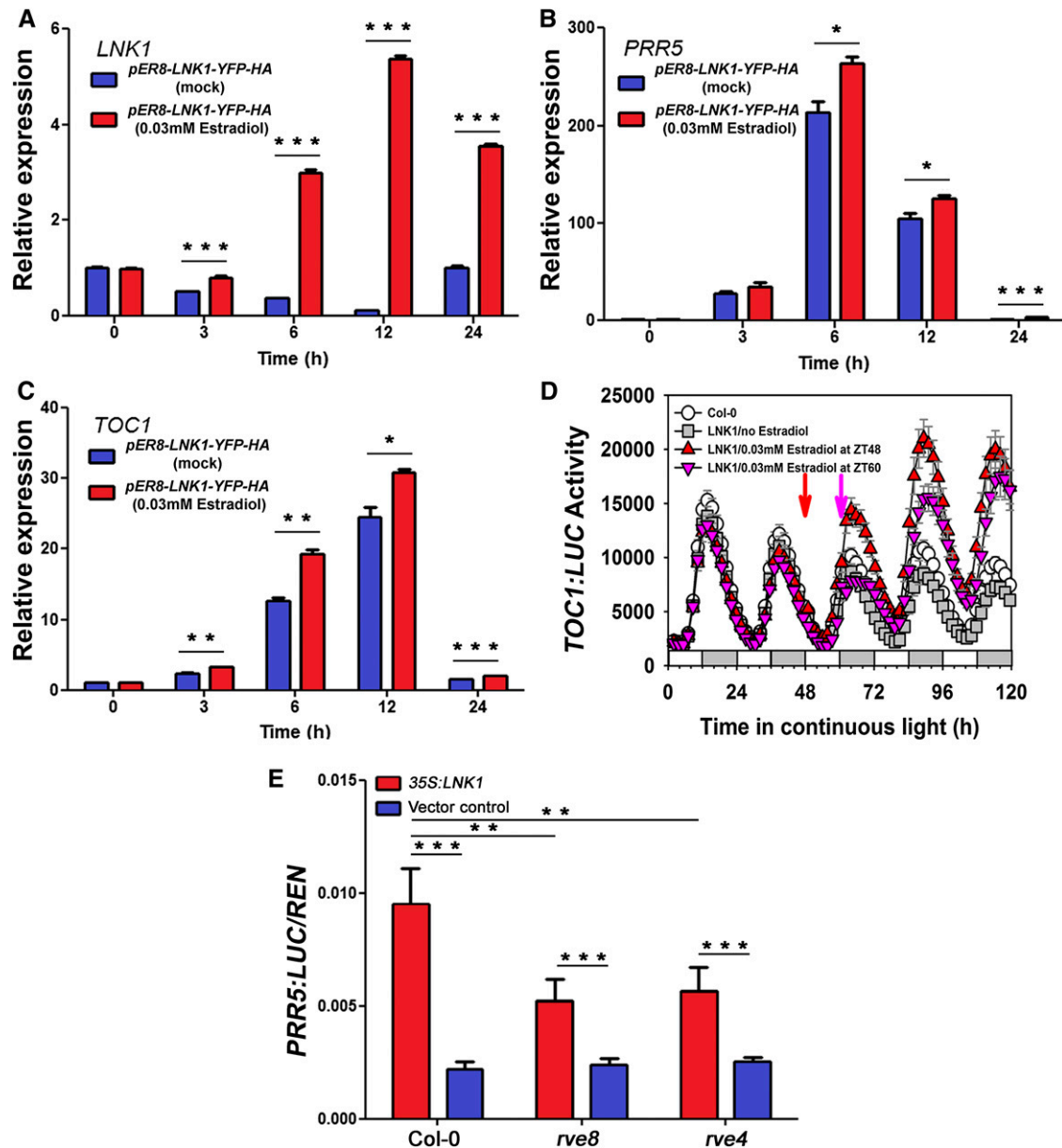


Figure 6. LNK1 Is a Transcriptional Coactivator of *PRR5* and *TOC1*.

(A) to (C) qRT-PCR detection of *LNK1* (A), *PRR5* (B), and *TOC1* (C) expression in lines carrying an estradiol-inducible version of *LNK1* (*pER8-LNK1-YFP-HA*). Seedlings were treated with DMSO (mock; blue bars) or estradiol (red bars) at ZT0, and RNA was isolated from tissue harvested at the indicated times. Horizontal lines indicate values that are significantly different (ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

(D) Effect of LNK1 induction on *TOC1:LUC* expression. Seedlings carrying an estradiol-inducible YFP/HA-tagged *LNK1* (*pER8-LNK1-YFP-HA*) were entrained under a 12-h-light/12-h-dark cycle and subsequently released to constant light conditions. LNK1 was induced by 30 μ M estradiol treatment at ZT48 (red arrow) or ZT60 (pink arrow), and luciferase activity was determined via a TopCount luminometer.

(E) Relative expression of *PRR5:LUC*, normalized to Renilla luciferase (*35S:RenLUC*), in transiently transfected *Arabidopsis* protoplasts constitutively overexpressing LNK1-FLAG in Col-0 and in *rve8* and *rve4* mutant backgrounds. Horizontal lines indicate values that are significantly different (ANOVA; ** $P < 0.01$, *** $P < 0.001$).

morning-phased genes, including *PRR9* and the *PHYTOCHROME INTERACTING FACTORS*, are repressed by the evening complex consisting of ELF3, ELF4, and the DNA binding protein LUX (also called PHYTOCLOCK1) (Helfer et al., 2011; Nusinow et al., 2011; Herrero et al., 2012). Transcription of the morning-

phased *CCA1* and *LHY* is repressed later in the day by the sequential binding of PRR9, PRR7, and PRR5 to the *CCA1* and *LHY* promoters (Nakamichi et al., 2010); this repression requires a corepressor, encoded by members of the *TPL/TPR* gene family (Wang et al., 2013).

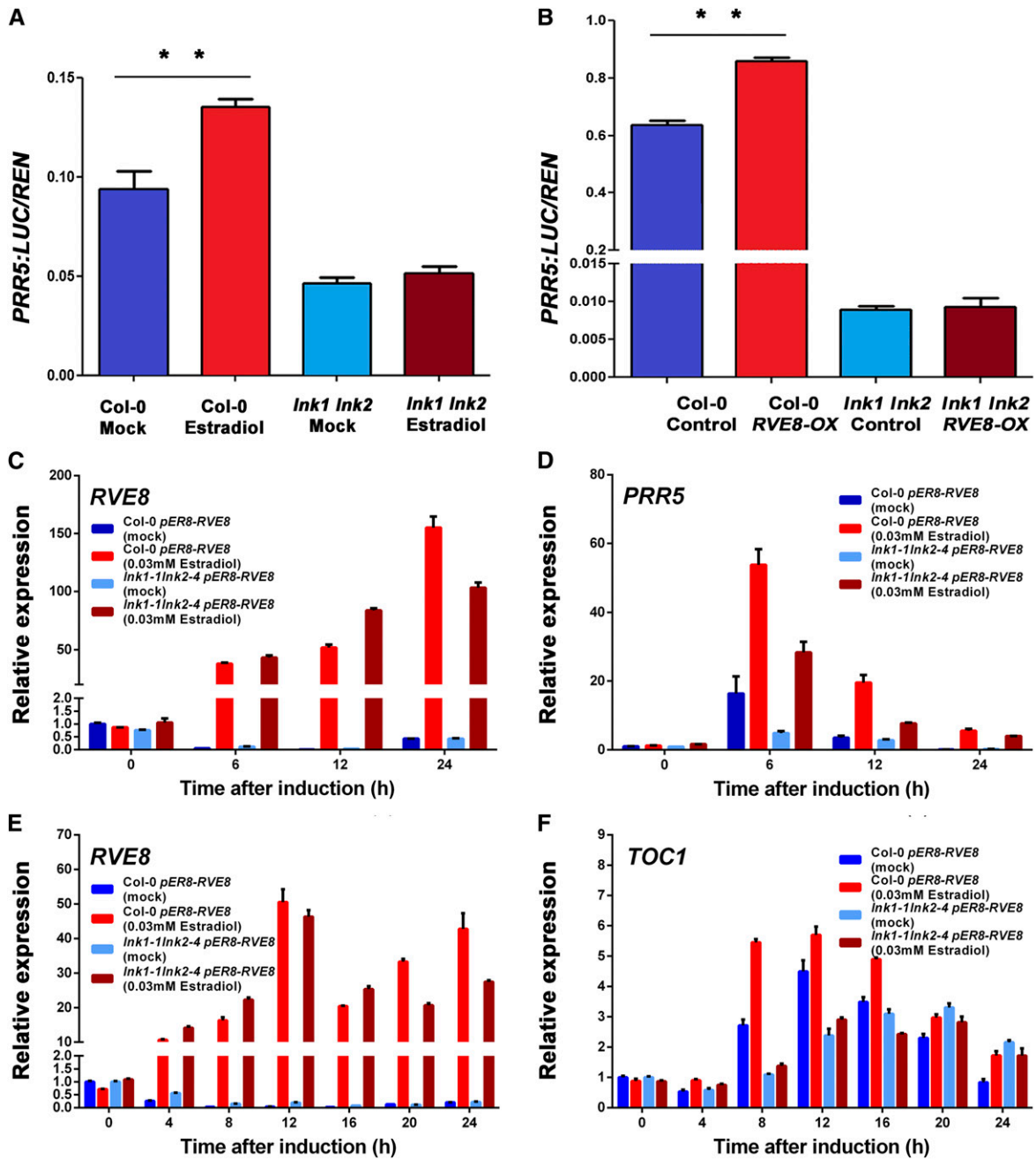


Figure 7. Full Activation of *PRR5* and *TOC1* Transcription by RVE8 Requires LNK1 and LNK2.

(A) and (B) Relative expression of *PRR5:LUC*, normalized to Renilla luciferase (*35S:RenLUC*), in transiently transfected *Arabidopsis* protoplasts expressing an estradiol-inducible RVE8 (A) or constitutively overexpressing RVE8 (B). Horizontal lines indicate values that are significantly different (ANOVA; **P < 0.01).

(C) and (D) qRT-PCR detection of *RVE8* (C) and *PRR5* (D) expression in mock-treated (blue and cyan bars) and estradiol-treated (red and purplish red bars) Col-0 and *Ink1 Ink2*, respectively, transgenic lines carrying an estradiol-inducible version of *RVE8*.

(E) and (F) qRT-PCR detection of *RVE8* (E) and *TOC1* (F) expression in mock-treated (blue and cyan bars) and estradiol-treated (red and purplish red bars) Col-0 and *Ink1 Ink2*, respectively, transgenic lines carrying an estradiol-inducible version of *RVE8*.

The data are presented as means \pm sd of three technical replicates.

Examples of transcriptional activation in the plant circadian clock are also accumulating. As indicated above, FHY3, FAR1, and HY5 activate the expression of *ELF4*. In mutants lacking function of both LIGHT-REGULATED WD1 (LWD1) and LWD2, the period is shortened and the expression of multiple clock genes is greatly reduced (Wu et al., 2008; Wang et al., 2011). LWD1 binds directly to the *PRR9*, *PRR5*, and *TOC1* promoters, suggesting that it is a transcriptional activator of these genes (Wang et al., 2011). The CCA1/LHY relatives RVE4, RVE6, and RVE8 have been defined as activating transcriptional factors in clock feedback loops (Hsu and Harmer, 2014). Morning-phased *RVE8* promotes the expression of evening-phased *PRR5* and *TOC1* as well as other evening components, including *LUX*, *ELF4*, and *GI*, by directly binding to the EE of their promoters (Farinas and Mas, 2011; Rawat et al., 2011; Hsu et al., 2013). Here, we report that LNK1 and LNK2 physically interact with RVE8 and RVE4 coincident with their morning-phased peak in expression. Transcriptional activation of *PRR5* or *TOC1* by RVE8 is attenuated in the *lnk1 lnk2* double mutant. Although the LNK proteins lack known DNA binding motifs and LNK1 fails to bind to the EE in vitro, LNK1 is recruited to EE-containing elements of the *PRR5* and *TOC1* promoters, as shown by chromatin immunoprecipitation. Therefore, we propose that LNK1, and by extension LNK2, serves as a transcriptional coactivator in a “morning complex” (or set of complexes) with the DNA binding proteins RVE8 and RVE4.

Our luciferase complementation data (Figures 4A and 4B; Supplemental Figure 5) suggest that the LNKs interact with the RVEs whenever the proteins are coexpressed, which is consistent with their common circadian phasing. However, there is considerable precedent for posttranslational modification of *Arabidopsis* clock proteins, and modifications such as phosphorylation are known to change the binding affinities of proteins for one another. For example, phosphorylation of *PRR5* and *TOC1* increases their affinity for the F-box protein ZTL (Fujiwara et al., 2008). It also is possible that other protein interactors may participate in these LNK/RVE complexes and might alter (either enhancing or attenuating) the affinities of LNK/RVE binding or perhaps sequester the LNKs from their RVE binding partners. ELF3 interaction with COP1 enables the interaction of COP1 with *GI* (Yu et al., 2008). When both *TOC1* and *PRR3* are highly phosphorylated, they interact and inhibit ZTL-*TOC1* binding (Fujiwara et al., 2008). Other modes of regulation by protein interaction could further modulate transcriptional activation by LNK/RVE complexes. For example, *ELF4* binds to *GI*, preventing the binding of *GI* to its DNA targets by subnuclear sequestration (Kim et al., 2013). *PRR5* regulates the phosphorylation and nuclear import of *TOC1*, which markedly modulates DNA binding and the repressor function of *TOC1* (Wang et al., 2010).

We also note that LNK1 and LNK2 each interacts with CCA1 and LHY as well as with RVE4 and RVE8. CCA1 and LHY, although best characterized as transcriptional repressors, have been implicated as activators of the three C-REPEAT BINDING FACTOR (CBF) genes (Seo et al., 2012) as well as of *PRR7* and *PRR9* (Farré et al., 2005). We speculate that the interaction of the LNKs with CCA1 and LHY may be sufficient to convert them from transcriptional repressors to transcriptional activators,

either simply by the recruitment of the LNK activation domain or possibly by concomitant masking of an intrinsic CCA1/LHY repressor domain or displacement of a bound corepressor.

Potential Mechanism of LNK in Circadian Transcriptional Control

The circadian clock of mammals, like that of plants, is an autoregulatory transcriptional network that consists of interlocked feedback loops. The core loop employs a heterodimer of two basic helix-loop-helix-PER-ARNT-SIM (PAS) transcription factors, CLOCK (CLK) or its brain-expressed paralog, NEURONAL PAS DOMAIN PROTEIN2, and BRAIN AND MUSCLE ARNT-LIKE1 (BMAL1), that activate transcription of the *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes during the day (Mohawk et al., 2012). Associated with the CLK-BMAL1 induction of target genes is rhythmic chromatin modification. CLK has intrinsic histone acetyltransferase (HAT) activity (Doi et al., 2006), and the CLK-BMAL heterodimer also recruits coactivators, including the CREB binding protein and P300, which also possess HAT activity (Koike et al., 2012; Aguilar-Arnal and Sassone-Corsi, 2013). Thus, CLK-BMAL1 binding mediates rhythmic chromatin reconfiguration, permitting the recruitment of additional clock-regulated transcription factors and allowing broad transcriptional programming (Menet et al., 2014).

Circadian transcriptional control in plants is known to include chromatin modification; *CCA1*, *LHY*, and *TOC1* transcription is positively correlated with levels of histone H3 acetylation and H3 Lys-4 trimethylation and inversely correlated with levels of H3 Lys-36 dimethylation (Hemmes et al., 2012; Malapeira et al.,

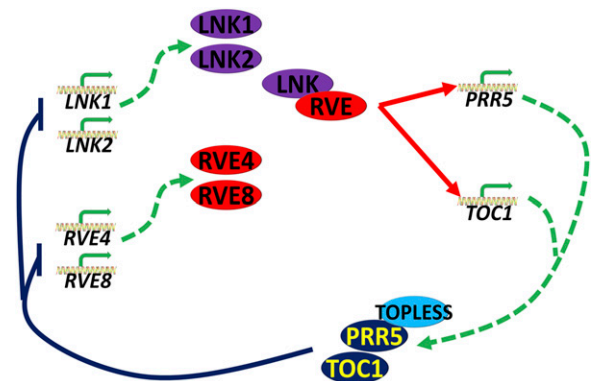


Figure 8. A Model Proposing a Role for the LNKs as Transcriptional Coactivators in the *Arabidopsis* Clock Feedback Loops of RVE4/RVE8 with PRR5 and TOC1.

LNK1 and LNK2 serve as transcriptional coactivators recruited to the promoters of target genes (here, *PRR5* and *TOC1*) via protein-protein interaction with the DNA binding transcription factors RVE8 and RVE4. Expression of *LNK1* and *LNK2* cycles in phase with *RVE4* and *RVE8* and peaks in mid morning. The LNK/RVE morning complex activates *PRR5* and *TOC1* transcription (red arrows). *TOC1* and a *PRR5*/TOP/TPL co-repressor complex feed back on the *LNK1*, *LNK2*, *RVE4*, and *RVE8* promoters to repress transcription (blue lines ending in perpendicular bars). Dashed green arrows represent transcription and translation.

[See online article for color version of this figure.]

2012; Song and Noh, 2012). RVE8 binding to the *TOC1* promoter is associated with increased histone H3 acetylation levels (Farinas and Mas, 2011). H3 Lys-4 trimethylation levels at the promoters of clock genes are increased by the histone methyltransferase SET DOMAIN GROUP2/*ARABIDOPSIS* TRITHORAX RELATED (SDG2/ATXR3) (Malapeira et al., 2012), but the identity of the responsible HAT(s) is not known (Henriques and Mas, 2013). LNK1 and LNK2 are plant-specific proteins that lack any recognized functional domains, so it is unlikely that they themselves have chromatin-modifying capacity. We postulate that LNK1 and LNK2 serve as coactivators connecting the clock-associated transcription factors CCA1, LHY, RVE4, and RVE8 to other components of the transcription complex, likely including chromatin-modifying complexes. Taken together, our results show that LNK1 serves as a transcriptional coactivator necessary for proper expression of the clock genes *PRR5* and *TOC1* through recruitment to their promoters via interaction with bona fide DNA binding proteins such as CCA1, LHY, RVE4, and RVE8 (Figure 8).

METHODS

Plant Materials and Growth Conditions

Plant materials used in this study were in the *Arabidopsis thaliana* Col-0 background, except *cca1-11* (Wassilewskija) and *lhy-21* (Wassilewskija). T-DNA insertion lines *lnk1-1* (SALK_024353), *lnk2-4* (CS807006), *lnk3-1* (SALK_085551C), and *lnk4-1* (CS120858) were obtained from the ABRC at Ohio State University. Seeds were sterilized in 20% bleach, placed on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) with 0.8% agar and 1% Suc, and then stratified for 3 d at 4°C in the dark. Plates were transferred to white light (70 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in a Percival CU36L5 growth chamber (Percival Scientific). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* was by floral dip (Clough and Bent, 1998).

Constructs

LNK1 and *LNK2* promoter-driven firefly *LUC* reporter plasmids were made through insertion of PCR-amplified (using Phusion High-Fidelity DNA Polymerase; New England Biolabs) promoter regions into a modified pENTR vector with the *LUC+* gene at multiple cloning sites. The *LNK1* and *LNK2* promoter regions (2157- and 1809-bp fragments before the start codon) were amplified from Col-0 genomic DNA with primer pairs LNK1-SA5_locus_F1/LNK1-SA5_Promoter_R and LNK2-CS1_locus_F1/LNK2-CS1_Promoter_R (Supplemental Table 4). The *promoter:LUC+* fragments were recombined into a modified pH2GW7 Δ (Karimi et al., 2002) from which the 35S promoter had been deleted via Gateway LR Clonase II enzyme (Life Technologies) (Supplemental Table 4).

To make *LNK1* and *LNK2* promoter-driven firefly *LUC* translational fusions to the *LNK* coding sequences (*LNK1:LNK1-LUC* and *LNK2:LNK2-LUC*), PCR products of full-length *LNK1* and *LNK2* genomic DNA including promoter and 3' untranslated region (UTR) were amplified from Col-0 genomic DNA with primer pairs LNK1-SA5_locus_F1/LNK1-SA5_locus_R2 and LNK2-CS1_locus_F1/LNK2-CS1_locus_R1 (Supplemental Table 4) and then cloned into pENTR. The *LNK* stop codons were then replaced with paired *SfiI* restriction sites through PCR amplification (primer pairs were LNK1-SA5-SF/LNK1-SA5-SR and LNK2-CS1-SF/LNK2-CS1-SR; Supplemental Table 4), and then a modified *LUC+* gene flanked by *SfiI* sites was inserted to create in-frame *LNK-LUC+* translational fusions (primers used to amplify *LUC+* were Luc+SF and Luc+SR; Supplemental Table 4). The resultant *LNK:LNK-LUC* fusions were then recombined into pH2GW7 Δ . The same strategy was used to make

the *PRR5pro:LUC+* plasmids and for the *LUC* complementation experiments. Full-length *LNK1/LNK2/CCA1/LHY/RVE4/RVE8* genomic DNAs were cloned into pENTR or pCR8 vectors and then modified to replace the stop codons with two *SfiI* sites between which either *nLUC* or *cLUC* was cloned to create in-frame translational fusions. The sense primers used for *nLUC* or *cLUC* amplification were Luc+SF and cLuc+SF, and the antisense primers were nLuc+SR and Luc+SR (Supplemental Table 4). The target genes were recombined into pH2GW7 Δ (Deleted 35S) or pMDC123 (Curtis and Grossniklaus, 2003). A related strategy was used to make the *PRR5:LUC+* transcriptional fusion, except that the entire *PRR5* coding sequence was replaced with paired *SfiI* sites (the *PRR5* promoter region used in this study was 3871 bp, and primers used to amplify the promoter were PRR5_locus_F and PRR5_NSF-NEW; Supplemental Table 4).

For gene expression pattern (*promoter:GUS*) assays, PCR-amplified *LNK* promoters were cloned into pMDC163 (*GUS*) (Curtis and Grossniklaus, 2003). The sense primers used in the amplification were LNK1-SA5_G_F and LNK2-CS1_G_F, and the antisense primers were LNK1-SA5_Prom_R and LNK2-CS1_Prom_R (Supplemental Table 4). For subcellular localization (C-terminal and N-terminal GFP fusions), PCR-amplified *LNK* promoter/coding sequences were cloned into pMDC43 (N-terminal GFP) (Curtis and Grossniklaus, 2003) or into *p35S:CDS-GFP* (C-terminal GFP with a pCAMBIA1300 [GenBank AF234296.1] vector backbone). The sense primers used in the amplification were LNK1-SA5_G_F and LNK2-CS1_G_F, and the antisense primers were LNK1-SA5_G_R_ns and LNK2-CS1_G_R_ns (Supplemental Table 4). Full-length coding sequences of *LNK1*, *LNK2*, *CCA1*, *LHY*, *RVE4*, and *RVE8* were cloned into pSPYNE-35S and pSPYCE-35S (Walter et al., 2004) for BiFC and into pCAMBIA-NLuc and pCAMBIA-CLuc (Chen et al., 2008) for *LUC* complementation.

To make *RVE8* overexpression and inducible constructs, *RVE8* cDNAs were amplified by PCR and inserted into pENTR/SD/D-TOPO (Invitrogen) and then transferred into pER8-GW (Papdi et al., 2008) or pMDC32 (Curtis and Grossniklaus, 2003).

LNK-GST and *CCA1-GST* fusion constructs were made by inserting PCR-amplified products into the *EcoRI* and *XhoI* sites of pGEX 4T-1 (GE Healthcare). PCR-amplified *LNK1* and *VP16* were inserted into pMN6 (Huq et al., 2004) at the *SmaI* and *KpnI* sites to test activator activity. pGLL was used as the reporter *LUC* and pRNL as the internal reference Renilla *LUC* reporter (Promega).

Gene Expression Assays

LNK1:GUS and *LNK2:GUS* transgenic (T3) lines were used to determine the expression pattern of *LNK1/LNK2* via histochemical *GUS* reporter activity (Jefferson et al., 1987). For RT-PCR analysis, total RNA was isolated from *Arabidopsis* samples using RNeasy Plus (TaKaRa) and treated with DNase I. After quantification of RNA, 3 μg of total RNA was used for cDNA synthesis using an oligo(dT) and RevertAid First Strand cDNA Synthesis Kit (Fermentas Thermo Fisher). TaKaRa SYBR Premix Ex Taq and a 7500 Fast Real-Time PCR instrument (Applied Biosystems) were used for qRT-PCR as described (Wang et al., 2012). *LNK1-FLAG* and *LNK1-YFP-HA* protein accumulation was assessed by immunoblot analysis. Protein extracts prepared from whole seedlings were separated on 8% SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore) by electroblotting. Immunoblot analysis was performed with monoclonal GFP or FLAG antibody (Sigma-Aldrich). Horseradish peroxidase-conjugated anti-mouse secondary antibody (Bio-Rad) was used to detect the primary antibody.

Protein-Protein Interaction

BiFC assays (Walter et al., 2004) were performed by coinfiltration of *Agrobacterium* carrying the N-terminal half of YFP (YFP^N) fused to *LNK1/*

LNK2 and the C-terminal half of YFP (YFP^C) fused to CCA1/LHY/RVE4/RVE8 vectors into *Nicotiana benthamiana* leaves. Confocal microscopy images were captured from epidermal cell layers of transfected leaves 2 to 3 d after infiltration. YFP and 4',6-diamidino-2-phenylindole (DAPI) fluorescence was observed by confocal microscopy: YFP/DAPI, excitation at 515/405 nm, emission at 525 to 560 nm/420 to 470 nm. For LNK-GFP fusion fluorescence: GFP/DAPI, excitation at 405 nm, emission at 505 to 530 nm/420 to 470 nm.

Firefly LCI and quantification were via transient expression of cLUC and nLUC fusions in *N. benthamiana* leaves via *Agrobacterium*-mediated coinfiltration as described (Chen et al., 2008). 35S overexpression fusion constructs in which the N-terminal half of firefly LUC (nLUC) was fused to LNK1/LNK2 and the C-terminal half (cLUC) was fused to the Myb transcription factor were coinfiltrated into *N. benthamiana* plants. Images were captured via a low-light cooled CCD camera (Andor Technology) 3 d after infiltration. A Packard TopCount luminometer (PerkinElmer) was used to quantify the LUC signal. Full-length gene fusion constructs in which cLUC was fused to LNK1/LNK2/CCA1 and nLUC was fused to (left to right) LNK2, CCA1, LHY, RVE8, or RVE4 were independently transformed into *Arabidopsis* to yield stable transgenic lines that were crossed for the measurement of LUC activity in F1 hybrids entrained to 12-h-light/12-h-dark cycles and transferred to constant light and temperature at ZT0. Data collected with a TopCount luminometer are presented as absolute LUC activity or normalized LUC activity, in which case the individual values were normalized to the average value for that trace. This facilitates the comparison of cycling between traces with different signal strengths.

Yeast two-hybrid analysis used a Gal4-based yeast hybrid system (Matchmaker two-hybrid system; Clontech). Full-length cDNAs of *LNK1/LNK2* and *RVE4/RVE8* were cloned into bait vector and prey vector (pGADT7 and pGBKT7). β -Galactosidase assays used the manufacturer's protocols.

Dual-Luciferase Transient Expression

Firefly LUC is driven by the minimal cauliflower mosaic virus 35S promoter Gal4 UAS in the pGLL reporter. Transfection efficiency was normalized by coinfection with 35S-driven Renilla luciferase. Expression driven by LNK1-Gal4-BD (LNK1) is relative to that of the effector plasmid (pMN6) alone. VP16 (Sadowski et al., 1988) served as a positive control. The Dual-Luciferase Reporter Assay System (Promega) was used to determine the relative expression of *PRR5:LUC*. *Arabidopsis* protoplasts were isolated from leaves of 4-week-old, soil-grown plants (entrained to 8-h-light/16-h-dark cycles at 22°C). Preparation of protoplasts (derived from the Col-0 wild type or the *lnk1 lnk2* double mutant) and subsequent polyethylene glycol-mediated transformation with reporter (*PRR5:LUC*), effector (*RVE8*, estradiol-inducible *RVE8* [*pER8-RVE8-YFP-HA*]), or constitutively overexpressing *RVE8* [*35S:RVE8*]), and internal control (*35S:RLUC*) plasmids were as described (Yoo et al., 2007). LUC activity was assayed using a Packard TopCount luminometer.

Firefly LUC Measurement and Data Analysis

Seedlings expressing the firefly luciferase reporter gene (*LUC*) under the control of promoters from *CCA1*, *LHY*, *TOC1* (Salomé and McClung, 2005b), and *LHCB1*1* (*CAB2*) (−199/+1) (Anderson et al., 1994) were entrained for 7 to 10 d in 12-h-light/12-h-dark cycles (22°C) before release into continuous light (22°C) conditions for LUC measurement with a TopCount luminometer. For temperature compensation assays, seedlings were entrained in 12-h-light/12-h-dark cycles at 22°C for 7 to 10 d before transfer to continuous light at 12, 22, and 27°C for LUC activity measurement. Circadian rhythms were assayed with BRASS 2.1.4, which employs fast Fourier transform nonlinear least squares (Plautz et al., 1997; Southern and Millar, 2005). The strength of a circadian rhythm is expressed as relative amplitude error (RAE). An ideal cosine wave is defined as RAE = 0, and RAE = 1 defines the statistically detectable limit of rhythmicity.

For fluence response curves, *CCA1:LUC* transgenic seedlings were entrained to 12-h-light/12-h-dark cycles for 7 d before transfer to constant red or blue light at the indicated fluence rates. On the first day in continuous light, seedlings were transferred to 96-well microplates (PerkinElmer) for LUC activity measurement; microplates were transferred manually to the Packard TopCount at 3-h intervals. The response of period to the fluence rate of constant red or blue light was analyzed by linear regression followed by analysis of covariance using GraphPad Prism software (<http://www.graphpad.com/>). For temperature compensation, Q_{10} was calculated as $Q_{10} = [\text{RateT2}/\text{RateT1}]^{10/(T2-T1)} = [(1/\text{period@T}_2)/(1/\text{period@T}_1)]^{10/(T2-T1)}$.

EMSA

EMSA were performed using biotin-labeled double-stranded probes and the Lightshift Chemiluminescent EMSA Kit (Pierce). Probes used in this study, including TOC1-EE-F, TOC1-EE-R, TOC1-mutEE-F, and TOC1-mutEE-R (Supplemental Table 4), were described previously (Harmer et al., 2000; Harmer and Kay, 2005; Pruneda-Paz et al., 2009).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed using 3-week-old seedlings stably transformed with pER8-LNK1-YFP-HA that were grown on Murashige and Skoog agar plates under 12-h-light/12-h-dark cycles at 22°C. According to the LCI results in stable *Arabidopsis* transgenic lines, the strongest interaction between LNK1 and RVE4/RVE8 is at ~ZT4, so leaf tissue samples were collected 28 h after induction with 30 μ M estradiol (Mizoi et al., 2006; Li et al., 2013) at ZT0. An EZ-ChIP Chromatin Immunoprecipitation Kit (Millipore) and an anti-GFP antibody (Roche) were used for chromatin immunoprecipitation assays. Primer pairs that amplified the indicated regions of *PRR5-EE*, *TOC1-EE*, *TOC1-3'UTR*, *ACTIN7*, and *UBIQUITIN10* (*UBQ10*) (Supplemental Table 4) were used to assess immunoprecipitation enrichment by PCR. All primer pairs were described previously (Pruneda-Paz et al., 2009; Rawat et al., 2011).

Accession Numbers

Sequence data for the genes described in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ACT7* (At5g09810), *CBF1* (At4g25490), *CBF2* (At4g25470), *CBF3* (At4g25480), *CCA1* (At2g46830), *CHE* (At5g08330), *COP1* (At2g32950), *DET1* (At4g10180), *ELF3* (At2g25930), *ELF4* (At2g40080), *FAR1* (At4g15090), *FHY3* (At3g22170), *GI* (At1g22770), *HY5* (At5g11260), *IPP2* (At3g02780), *LHCB1*1* (At1g29920), *LHY* (At1g01060), *LNK1* (At5g64170), *LNK2* (At3g54500), *LNK3* (At3g12320), *LNK4* (At5g06980), *LUX* (At3g46640), *LWD1* (At1g12910), *LWD2* (At3g26640), *TPL/TPR* (At1g15750, At1g80490, At3g16830, At5g27030, and At3g15880), *PRR3* (At5g60100), *PRR5* (At5g24470), *PRR7* (At5g02810), *PRR9* (At2g46790), *RVE4* (At5g02840), *RVE8* (At3g09600), *SDG2/ATXR3* (At4g15180), *TOC1* (At5g61380), *UBQ10* (At4g05320), and *ZTL* (At5g57360).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *LNK1* and *LNK2* Are Required for Circadian Period Determination.

Supplemental Figure 2. Accumulation of *LNK1* mRNA and Protein in Lines Constitutively or Inducibly Overexpressing *LNK1*.

Supplemental Figure 3. *LNK1* and *LNK2* Expression in *Arabidopsis* Seedlings.

Supplemental Figure 4. Subcellular Localization of *LNK-GFP* Fusion Proteins.

Supplemental Figure 5. Quantification of Luciferase Complementation Imaging Assays.

Supplemental Figure 6. Yeast Two-Hybrid Analysis of LNK1 and LNK2 Interaction with RVE4, RVE8, PRR7, and PRR9.

Supplemental Figure 7. Effect of Slight *CCA1* Overexpression on Circadian Period Length.

Supplemental Figure 8. Expression (mRNA Abundance) Analysis of *LNK1*, *LNK2*, *CCA1*, *LHY*, *RVE4*, and *RVE8* in Circadian Free Run.

Supplemental Figure 9. Protein Stability of LNK1 and Mutated m1-LNK1.

Supplemental Table 1. Summary of Effects of Perturbed *LNK* Expression on Circadian Gene Expression.

Supplemental Table 2. Effect of Loss of *LNK* Function on Light Sensitivity of the Circadian Clock (Figures 1G and 1H).

Supplemental Table 3. Effect of Loss of *LNK* Function on Temperature Sensitivity of the Clock (Figure 1I).

Supplemental Table 4. Oligonucleotides (Shown 5' → 3') Used in This Study.

ACKNOWLEDGMENTS

We thank Jun-Xian He for the *pMN6* transient assay system and Bingchun Zhao and Rui Li for technical help with *Arabidopsis* mesophyll protoplast preparation and transient expression. This work was supported by grants from the Special Program for Key Basic Research of the Ministry of Science and Technology of China (Grant 2012CB126303), the National Science Foundation of China (Grant 31071247), the Program for New Century Excellent Talents in University of the Ministry of Education of China (Grant NCET-13-0771), and the Hebei Science Fund for Distinguished Young Scholars (Grants C2011205034, CPRC036, and 20100502) to X.X. and from the U.S. National Science Foundation (Grants IOS-0923752 and IOS-1025965) to C.R.M.

AUTHOR CONTRIBUTIONS

Q.X. and X.X. conceived the project and designed the experiments. Q.X. and P.W. generated the constructs. Q.X., X.X., X.L., L.W., P.W., L.Y., Y.L., and Z.Y. screened the mutants, generated the transgenic and hybrid lines, and characterized the circadian phenotype in various conditions. P.W., Q.X., H.X., and C.Z. performed the LCI analysis. P.W. and C.Z. performed the BiFC, chromatin immunoprecipitation, EMSA, and dual-luciferase transient expression in protoplasts. X.L. analyzed the clock gene expression with qRT-PCR. P.W. and X.L. generated yeast two-hybrid plasmids, and X.L. performed the β -galactosidase analysis. X.X., L.Y., Y.L., and L.Z. did the fluence response curves assay. Q.X., C.R.M., and X.X. wrote the article.

Received April 17, 2014; revised June 8, 2014; accepted June 20, 2014; published July 10, 2014.

REFERENCES

- Aguilar-Arnal, L., and Sassone-Corsi, P.** (2013). The circadian epigenome: How metabolism talks to chromatin remodeling. *Curr. Opin. Cell Biol.* **25**: 170–176.
- Anderson, S.L., Teakle, G.R., Martino-Catt, S.J., and Kay, S.A.** (1994). Circadian clock- and phytochrome-regulated transcription is conferred by a 78 bp *cis*-acting domain of the *Arabidopsis* *CAB2* promoter. *Plant J.* **6**: 457–470.
- Carré, I.A., and Kim, J.-Y.** (2002). MYB transcription factors in the *Arabidopsis* circadian clock. *J. Exp. Bot.* **53**: 1551–1557.
- Chen, H., Zou, Y., Shang, Y., Lin, H., Wang, Y., Cai, R., Tang, X., and Zhou, J.M.** (2008). Firefly luciferase complementation imaging assay for protein-protein interactions in plants. *Plant Physiol.* **146**: 368–376.
- Clough, S.J., and Bent, A.F.** (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**: 735–743.
- Curtis, M.D., and Grossniklaus, U.** (2003). A Gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* **133**: 462–469.
- Dodd, A.N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., Hibberd, J.M., Millar, A.J., and Webb, A.A.R.** (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309**: 630–633.
- Doi, M., Hirayama, J., and Sassone-Corsi, P.** (2006). Circadian regulator CLOCK is a histone acetyltransferase. *Cell* **125**: 497–508.
- Dunlap, J.C.** (1999). Molecular bases for circadian clocks. *Cell* **96**: 271–290.
- Farinas, B., and Mas, P.** (2011). Functional implication of the MYB transcription factor RVE8/LCL5 in the circadian control of histone acetylation. *Plant J.* **66**: 318–329.
- Farré, E.M., Harmer, S.L., Harmon, F.G., Yanovsky, M.J., and Kay, S.A.** (2005). Overlapping and distinct roles of PRR7 and PRR9 in the *Arabidopsis* circadian clock. *Curr. Biol.* **15**: 47–54.
- Fujiwara, S., Wang, L., Han, L., Suh, S.S., Salomé, P.A., McClung, C.R., and Somers, D.E.** (2008). Post-translational regulation of the *Arabidopsis* circadian clock through selective proteolysis and phosphorylation of pseudo-response regulator proteins. *J. Biol. Chem.* **283**: 23073–23083.
- Gendron, J.M., Pruneda-Paz, J.L., Doherty, C.J., Gross, A.M., Kang, S.E., and Kay, S.A.** (2012). *Arabidopsis* circadian clock protein, TOC1, is a DNA-binding transcription factor. *Proc. Natl. Acad. Sci. USA* **109**: 3167–3172.
- Graf, A., and Smith, A.M.** (2011). Starch and the clock: the dark side of plant productivity. *Trends Plant Sci.* **16**: 169–175.
- Harmer, S.L., and Kay, S.A.** (2005). Positive and negative factors confer phase-specific circadian regulation of transcription in *Arabidopsis*. *Plant Cell* **17**: 1926–1940.
- Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A.** (2000). Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* **290**: 2110–2113.
- Helper, A., Nusinow, D.A., Chow, B.Y., Gehrke, A.R., Bulyk, M.L., and Kay, S.A.** (2011). *LUX ARRHYTHMO* encodes a nighttime repressor of circadian gene expression in the *Arabidopsis* core clock. *Curr. Biol.* **21**: 126–133.
- Hemmes, H., Henriques, R., Jang, I.-C., Kim, S., and Chua, N.-H.** (2012). Circadian clock regulates dynamic chromatin modifications associated with *Arabidopsis* *CCA1/LHY* and *TOC1* transcriptional rhythms. *Plant Cell Physiol.* **53**: 2016–2029.
- Henriques, R., and Mas, P.** (2013). Chromatin remodeling and alternative splicing: Pre- and post-transcriptional regulation of the *Arabidopsis* circadian clock. *Semin. Cell Dev. Biol.* **24**: 399–406.
- Herrero, E., et al.** (2012). EARLY FLOWERING4 recruitment of EARLY FLOWERING3 in the nucleus sustains the *Arabidopsis* circadian clock. *Plant Cell* **24**: 428–443.
- Hsu, P.Y., and Harmer, S.L.** (2014). Wheels within wheels: the plant circadian system. *Trends Plant Sci.* **19**: 240–249.
- Hsu, P.Y., Devisetty, U.K., and Harmer, S.L.** (2013). Accurate timekeeping is controlled by a cycling activator in *Arabidopsis*. *eLife* **2**: e00473.
- Huang, W., Pérez-García, P., Pokhilko, A., Millar, A.J., Antoshechkin, I., Riechmann, J.L., and Mas, P.** (2012). Mapping the core of the *Arabidopsis* circadian clock defines the network structure of the oscillator. *Science* **336**: 75–79.

- Huq, E., Al-Sady, B., Hudson, M., Kim, C., Apel, K., and Quail, P.H. (2004). Phytochrome-interacting factor 1 is a critical bHLH regulator of chlorophyll biosynthesis. *Science* **305**: 1937–1941.
- James, A.B., Monreal, J.A., Nimmo, G.A., Kelly, C.L., Herzyk, P., Jenkins, G.I., and Nimmo, H.G. (2008). The circadian clock in *Arabidopsis* roots is a simplified slave version of the clock in shoots. *Science* **322**: 1832–1835.
- Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β -Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**: 3901–3907.
- Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.* **7**: 193–195.
- Kim, Y., Lim, J., Yeom, M., Kim, H., Kim, J., Wang, L., Kim, W.Y., Somers, D.E., and Nam, H.G. (2013). ELF4 regulates GIGANTEA chromatin access through subnuclear sequestration. *Cell Reports* **3**: 671–677.
- Koike, N., Yoo, S.H., Huang, H.C., Kumar, V., Lee, C., Kim, T.K., and Takahashi, J.S. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* **338**: 349–354.
- Lau, O.S., Huang, X., Charron, J.-B., Lee, J.-H., Li, G., and Deng, X.W. (2011). Interaction of *Arabidopsis* DET1 with CCA1 and LHY in mediating transcriptional repression in the plant circadian clock. *Mol. Cell* **43**: 703–712.
- Li, G., et al. (2011). Coordinated transcriptional regulation underlying the circadian clock in *Arabidopsis*. *Nat. Cell Biol.* **13**: 616–622.
- Li, Z., Peng, J., Wen, X., and Guo, H. (2013). Ethylene-insensitive3 is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in *Arabidopsis*. *Plant Cell* **25**: 3311–3328.
- Malapeira, J., Khaitova, L.C., and Mas, P. (2012). Ordered changes in histone modifications at the core of the *Arabidopsis* circadian clock. *Proc. Natl. Acad. Sci. USA* **109**: 21540–21545.
- McClung, C.R. (2014). Wheels within wheels: New transcriptional feedback loops in the *Arabidopsis* circadian clock. *F1000Prime Rep.* **6**: 2.
- Menet, J.S., Pescatore, S., and Rosbash, M. (2014). CLOCK:BMAL1 is a pioneer-like transcription factor. *Genes Dev.* **28**: 8–13.
- Mizoi, J., Nakamura, M., and Nishida, I. (2006). Defects in CTP: PHOSPHORYLETHANOLAMINE CYTIDYLTRANSFERASE affect embryonic and postembryonic development in *Arabidopsis*. *Plant Cell* **18**: 3370–3385.
- Mohawk, J.A., Green, C.B., and Takahashi, J.S. (2012). Central and peripheral circadian clocks in mammals. *Annu. Rev. Neurosci.* **35**: 445–462.
- Murashige, T.R., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15**: 473–497.
- Nakamichi, N., Kiba, T., Henriques, R., Mizuno, T., Chua, N.-H., and Sakakibara, H. (2010). PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the *Arabidopsis* circadian clock. *Plant Cell* **22**: 594–605.
- Nusinow, D.A., Helfer, A., Hamilton, E.E., King, J.J., Imaizumi, T., Schultz, T.F., Farré, E.M., and Kay, S.A. (2011). The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. *Nature* **475**: 398–402.
- Papdi, C., Abrahám, E., Joseph, M.P., Popescu, C., Koncz, C., and Szabados, L. (2008). Functional identification of *Arabidopsis* stress regulatory genes using the controlled cDNA overexpression system. *Plant Physiol.* **147**: 528–542.
- Plautz, J.D., Straume, M., Stanewsky, R., Jamison, C.F., Brandes, C., Dowse, H.B., Hall, J.C., and Kay, S.A. (1997). Quantitative analysis of *Drosophila period* gene transcription in living animals. *J. Biol. Rhythms* **12**: 204–217.
- Pokhilko, A., Fernández, A.P., Edwards, K.D., Southern, M.M., Halliday, K.J., and Millar, A.J. (2012). The clock gene circuit in *Arabidopsis* includes a repressilator with additional feedback loops. *Mol. Syst. Biol.* **8**: 574.
- Portolés, S., and Más, P. (2010). The functional interplay between protein kinase CK2 and CCA1 transcriptional activity is essential for clock temperature compensation in *Arabidopsis*. *PLoS Genet.* **6**: e1001201.
- Pruneda-Paz, J.L., Breton, G., Para, A., and Kay, S.A. (2009). A functional genomics approach reveals CHE as a component of the *Arabidopsis* circadian clock. *Science* **323**: 1481–1485.
- Rawat, R., Takahashi, N., Hsu, P.Y., Jones, M.A., Schwartz, J., Salemi, M.R., Phinney, B.S., and Harmer, S.L. (2011). REVEILLE8 and PSEUDO-RESPONSE REGULATOR5 form a negative feedback loop within the *Arabidopsis* circadian clock. *PLoS Genet.* **7**: e1001350.
- Rugnone, M.L., Faigón Soverna, A., Sanchez, S.E., Schlaen, R.G., Hernando, C.E., Seymour, D.K., Mancini, E., Chernomoretz, A., Weigel, D., Más, P., and Yanovsky, M.J. (2013). LNK genes integrate light and clock signaling networks at the core of the *Arabidopsis* oscillator. *Proc. Natl. Acad. Sci. USA* **110**: 12120–12125.
- Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**: 563–564.
- Salomé, P.A., and McClung, C.R. (2005a). What makes the *Arabidopsis* clock tick on time? A review on entrainment. *Plant Cell Environ.* **28**: 21–38.
- Salomé, P.A., and McClung, C.R. (2005b). PSEUDO-RESPONSE REGULATOR 7 and 9 are partially redundant genes essential for the temperature responsiveness of the *Arabidopsis* circadian clock. *Plant Cell* **17**: 791–803.
- Seo, P.-J., Park, M.-J., Lim, M.-H., Kim, S.-G., Lee, M., Baldwin, I.T., and Park, C.-M. (2012). A self-regulatory circuit of CIRCADIAN CLOCK-ASSOCIATED1 underlies the circadian clock regulation of temperature responses in *Arabidopsis*. *Plant Cell* **24**: 2427–2442.
- Song, H.-R., and Noh, Y.-S. (2012). Rhythmic oscillation of histone acetylation and methylation at the *Arabidopsis* central clock loci. *Mol. Cells* **34**: 279–287.
- Southern, M.M., and Millar, A.J. (2005). Circadian genetics in the model higher plant, *Arabidopsis thaliana*. *Methods Enzymol.* **393**: 23–35.
- Walter, M., Chaban, C., Schütze, K., Batistic, O., Weckermann, K., Näge, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K., and Kudla, J. (2004). Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* **40**: 428–438.
- Wang, L., Fujiwara, S., and Somers, D.E. (2010). PRR5 regulates phosphorylation, nuclear import and subnuclear localization of TOC1 in the *Arabidopsis* circadian clock. *EMBO J.* **29**: 1903–1915.
- Wang, L., Kim, J., and Somers, D.E. (2013). Transcriptional corepressor TOPLESS complexes with pseudoresponse regulator proteins and histone deacetylases to regulate circadian transcription. *Proc. Natl. Acad. Sci. USA* **110**: 761–766.
- Wang, X., et al. (2012). SKIP is a component of the spliceosome linking alternative splicing and the circadian clock in *Arabidopsis*. *Plant Cell* **24**: 3278–3295.
- Wang, Y., Wu, J.-F., Nakamichi, N., Sakakibara, H., Nam, H.-G., and Wu, S.-H. (2011). LIGHT-REGULATED WD1 and PSEUDO-RESPONSE REGULATOR9 form a positive feedback regulatory loop in the *Arabidopsis* circadian clock. *Plant Cell* **23**: 486–498.
- Wu, J.-F., Wang, Y., and Wu, S.-H. (2008). Two new clock proteins, LW1 and LW2, regulate *Arabidopsis* photoperiodic flowering. *Plant Physiol.* **148**: 948–959.
- Yoo, S.-D., Cho, Y.-H., and Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2**: 1565–1572.
- Yu, J.-W., et al. (2008). COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. *Mol. Cell* **32**: 617–630.
- Zhang, E.E., and Kay, S.A. (2010). Clocks not winding down: Unravelling circadian networks. *Nat. Rev. Mol. Cell Biol.* **11**: 764–776.