LNK genes integrate light and clock signaling networks at the core of the *Arabidopsis* oscillator

Matias L. Rugnone[a], Ana Faigón Soverna[a], Sabrina E. Sanchez[a,1], Ruben Gustavo Schlaen[a,1], Carlos Esteban Hernando[a,1], Danelle K. Seymour[b], Estefanía Mancini[a], Ariel Chernomoretz[a], Detlef Weigel[b], Paloma Más[c], and Marcelo J. Yanovsky[a,2]

[a]Fundación Instituto Leloir, Instituto de Investigaciones Bioquímicas de Buenos Aires–Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, C1405BWE Buenos Aires, Argentina; [b]Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany; and [c]Molecular Genetics Department, Center for Research in Agricultural Genomics (CRAG), Parc de Recerca, Universitat Autònoma de Barcelona, Edifici CRAG, Barcelona 08193, Spain

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Light signaling pathways and the circadian clock interact to help organisms synchronize physiological and developmental processes with periodic environmental cycles. The plant photoreceptors responsible for clock resetting have been characterized, but signaling components that link the photoreceptors to the clock remain to be identified. Here we describe a family of night light–inducible and clock-regulated genes (*LNK*) that play a key role linking light regulation of gene expression to the control of daily and seasonal rhythms in *Arabidopsis thaliana*. A genomewide transcriptome analysis revealed that most light-induced genes respond more strongly to light during the subjective day, which is consistent with the diurnal nature of most physiological processes in plants. However, a handful of genes, including the homologous genes *LNK1* and *LNK2*, are more strongly induced by light in the middle of the night, when the clock is most responsive to this signal. Further analysis revealed that the morning phased *LNK1* and *LNK2* genes control circadian rhythms, photomorphogenic responses, and photoperiodic dependent flowering, most likely by regulating a subset of clock and flowering time genes in the afternoon. *LNK1* and *LNK2* themselves are directly repressed by members of the *TIMING OF CAB1 EXPRESSION/PSEUDO RESPONSE REGULATOR* family of core-clock genes in the afternoon and early night. Thus, *LNK1* and *LNK2* integrate early light signals with temporal information provided by core oscillator components to control the expression of afternoon genes, allowing plants to keep track of seasonal changes in day length.

The rotation of the earth around its own axis and its movement around the sun cause daily and seasonal oscillations in light intensity on our planet. The profound impact of these environmental changes on biological processes strongly contributed to the evolution of circadian clocks (1). Therefore, it is not surprising that circadian and light signaling networks are intimately connected. Indeed, although circadian rhythms normally persist in the absence of environmental cues with a period of ∼24 h, light/dark cycles entrain the clock and thereby ensure appropriate phasing of circadian rhythms in relation to changing sunrise and sunset throughout the year (2).

In plants, the effect of light on the clock is mediated by specific photoreceptors, such as phytochromes, cryptochromes, and members of the ZEITLUPE protein family (3–5). The plant circadian clock is mostly based on clock genes that mutually regulate each other expression (6), and some of these are acutely induced by phytochromes (7–9). Interestingly, cryptochromes and phytochromes are not essential for circadian oscillations in *Arabidopsis* plants (10–12), but circadian regulation of phototransduction pathways generates tight links between these two signaling networks (13). This phenomenon, known as gating, was originally described for the light-regulated activity of the promoter of the *CHLOROPHYLL A/B BINDING PROTEIN II* (*CABII*) gene (14). *CABII* expression is acutely induced by red light pulses, but the effectiveness of this treatment oscillates during a 24-h day, with maximal effects when photosynthetic activity is expected to be at its peak during the day and minimal effects during the night (14–16). Clock gating of light signaling is mediated, at least in part, by the clock gene *EARLY FLOWERING 3* (15), which interacts directly with phytochrome B (17). Clock regulation of light signaling also influences physiological processes such stem elongation (18, 19), and the clock itself (15, 20). Indeed, in plants grown under light/dark cycles and then transferred to constant darkness brief light pulses are most effective in resetting the phase of circadian rhythms during the night rather than during the subjective day (i.e., the phase that would have been illuminated if the plants were kept under light/dark cycles) (20). This phenomenon is shared across kingdoms, suggesting that it is critical for the appropriate adjustment of circadian rhythms to the environment (21).

Despite the importance of the interactions between light and the circadian clock in the control of biological activities in plants, a comprehensive analysis of these interactions has been lacking. Light signaling and circadian networks operate primarily by transcriptional control (13, 22–24). To characterize these interactions in *Arabidopsis*, we evaluated the response of the *Arabidopsis* transcriptome to light pulses given at different times. A light pulse in the middle of the subjective day should modulate the expression of genes that contribute to maximizing process such as photosynthesis. In contrast, a light pulse in the middle of the night simulates either an earlier sunrise or a later sunset and may reveal genes involved in clock resetting and/or seasonal adjustment. Indeed, this analysis allowed us to identify a unique family of light and clock regulated morning genes. These genes control both the pace of circadian rhythms and the photoperiodic regulation of flowering time, apparently by promoting the expression of a subset of core-clock and clock-output genes in the afternoon.

Results

Light Treatments Are More Effective During the Subjective Day. To investigate if and how time of day affects light regulation of gene expression at a global level, we used microarray analysis to

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[1]S.E.S., R.G.S., and C.E.H. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: myanovsky@leloir.org.ar.

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evaluate the response of the *Arabidopsis* transcriptome to a 1-h light treatment given either in the middle of the subjective day or in the middle of the night (Fig. 1*A*). Many light-regulated genes showed a stronger response to a light pulse given during the subjective day compared with a similar treatment given during the night (Fig. 1*B*; Dataset S1). Among a total of 2,237 light-induced genes identified using a twofold change as cutoff, 1,537 responded at least twice as strongly to the light pulse given in the middle of the subjective day (Dataset S1*A*), and only 65 genes showed a stronger response during the night (Dataset S1*B*). Thus, almost 70% of light-induced genes behaved similarly to what had been reported for *CABII* (14). This group of day light–responsive genes was enriched in gene ontologies associated with metabolism, chloroplast components, responses to environmental stimuli, and responses to abiotic and biotic stress (Dataset S2*A*). The influence of time of day was less pronounced for light-repressed genes. Among a total of 1,672 light-repressed genes, only 607 responded at least twice as much during the subjective day compared with the night (Dataset S1*C*), and 78 showed the opposite response (Dataset S1*D*). The group more strongly repressed by light during the subjective day was mostly enriched in genes involved in amino acid catabolism (Dataset S2*B*), whereas those more responsive to light during the night were associated with hormonal regulation, among other processes (Dataset S2*C*).

Because plants were under starvation during the subjective day, the effect of light at this time of day could simply be the consequence of sucrose reaccumulation due to photosynthetic activity. However, no significant correlation was found between light induction of gene expression during the subjective day and changes in gene expression induced by sucrose or enhanced photosynthetic activity (Fig. S1). In contrast, a direct correlation was found for light-repressed genes (Fig. S1). Indeed, using quantitative RT-PCR (qRT-PCR), we found that light repression of two genes was unaffected in photoreceptor mutants, whereas light induction was significantly attenuated in *phyA;phyB* mutants and to a lesser extent in *cry1;cry2* mutants (Fig. S2). In addition, the expression of these light-induced genes is not affected by sucrose or photosynthetic activity, whereas light-repressed genes were also repressed to some extent by sucrose or photosynthetic activity (Fig. S2). Thus, light induction of gene expression during the subjective day is mostly mediated by photomorphogenic photoreceptors, whereas repression is likely triggered by sucrose accumulation due to photosynthetic activity.

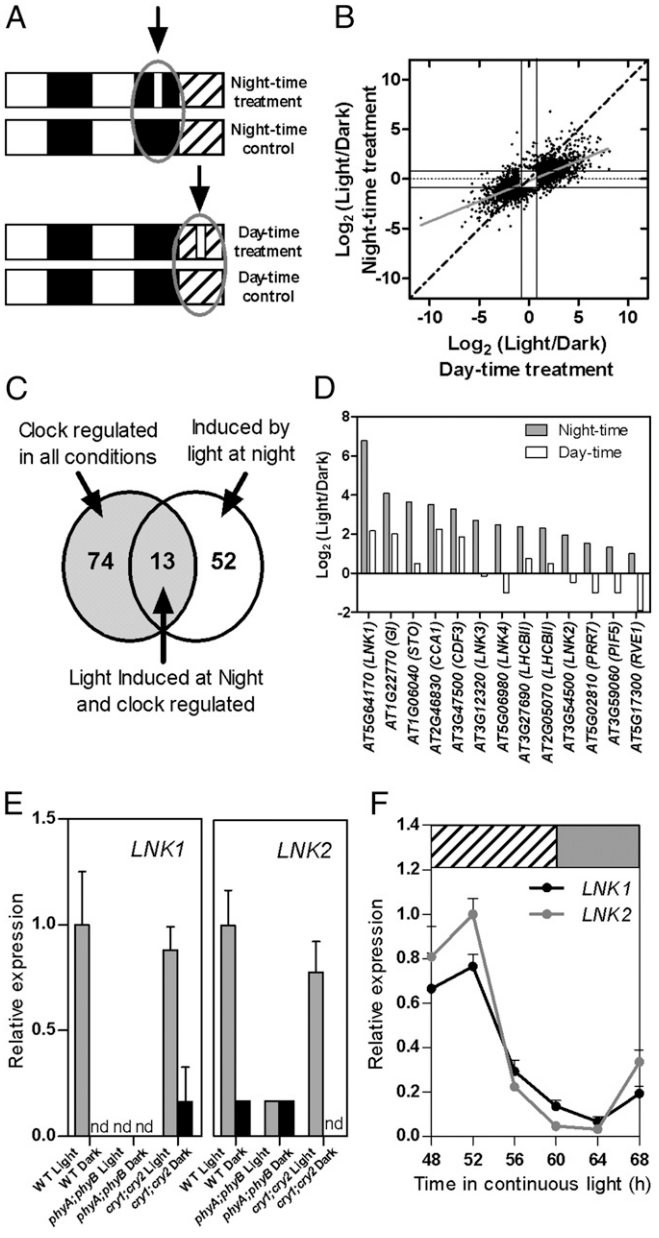

Fig. 1. Genomewide analysis of light and clock interactions in the control of gene expression and identification of *LNK* genes. (*A*) Experimental design. Plants were grown under 12-h light/12-h dark cycles for 14 d and then exposed or not to a 1-h light pulse in the middle of the night or subjective day on the 15th day. (*B*) Comparative genomewide expression analysis of the effect of a light pulse given during subjective day time (*x* axis) vs. night time (*y* axis). (*C*) Overlap between 87 genes that are rhythmically expressed under multiple conditions (23) and 65 genes that showed a stronger induction by light during night time compared with subjective day time (Dataset S1). (*D*) Microarray data corresponding to the relative response of *LNK* genes to a 1-h light treatment given in the middle of the night or subjective day. (*E*) Relative expression levels of *LNK1* and *LNK2* measured by qRT-PCR. The analysis was conducted in WT, *phyA;phyB*, and *cry;cry2* plants grown under 12-h light/12-h dark cycles and exposed or not to a 1-h light pulse in the middle of the night ($n = 3$). nd, not detectable. Data represent average + SEM. (*F*) Circadian expression of *LNK1* and *LNK2* genes. Expression was determined by qRT-PCR during the third day under free running conditions. $n = 4$. Data are average + SEM. White, dark, gray, and hatched boxes indicate day, night, subjective night, and subjective day, respectively.

Night Light Is More Effective in Inducing the Expression of a Subset of Core-Clock Genes. Clock entrainment is most sensitive to light pulses given during the night, a treatment that simulates seasonal changes in day length (20). Consistent with this, the subset of 65 genes responding at least twice as strongly to the night light treatment was significantly enriched in clock genes, a phenomenon that was specific for this particular class of light-regulated genes (Dataset S2*D*). Clock genes are also enriched among those with oscillations that are robust to different experimental conditions, such as continuous light, continuous darkness, short days, long days, temperature cycles, etc. (23). Thus, we reasoned that the list of genes that are more effectively induced by night light and also cycle under multiple conditions should contain new candidate clock regulators. Thirteen genes fulfilled both criteria, a 30-fold enrichment over expectation ($P < 1 \times 10^{-15}$, hypergeometric distribution; Fig. 1*C*). This group included the clock genes *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *PRR7*, and *GIGANTEA* (*GI*), six genes involved in the control of stem elongation, flowering time or photosynthesis, as well as four genes that constitute a new family of plant specific proteins, which we named *LNK1–4*, for night light–inducible and clock-regulated genes 1–4 (Fig. 1*D*).

LNK1 (AT5G64170) and LNK2 (AT3G54500) are proteins of about 66 kDa, with 35% sequence similarity across their length. LNK3 (AT3G12320) and LNK4 (AT5G06980) proteins are smaller (each around 30 kDa), with 60% sequence similarity and

with a third of conserved positions also shared with LNK1/LNK2 (Fig. S3). *LNK* homologs can be found throughout land plants, including nonvascular plants. *LNK3* and *LNK4* appear to be the result of a recent duplication event within the Brassicaceae (Fig. S4). Because *LNK1* responded most strongly to the night light treatment (Dataset S1*B*), we focused on *LNK1* and its closest homolog, *LNK2*. qRT-PCR analyses of WT and mutants indicated that these two genes are induced by a light pulse in the middle of the night via the phytochrome family of red/far-red light photoreceptors and that they are rhythmically expressed with maximum levels in the subjective morning (Fig. 1 *E* and *F*).

LNK1 and LNK2 Regulate Light Signaling and Biological Timing. To determine whether *LNK1* and *LNK2* affect light- and clock-regulated developmental and physiological processes, several mutants with T-DNA insertions in these two genes were identified and characterized in detail (Fig. S5). An early developmental phenomenon under control of light and the circadian clock is the elongation of the hypocotyl, the embryonic stem. No significant differences in hypocotyl length were observed among WT plants and *lnk1*, *lnk2*, or *lnk1;lnk2* mutants grown in complete darkness (Fig. S6*A*). In contrast, *lnk1* mutants had longer hypocotyls than WT plants under continuous white light (Fig. 2*A*; Fig. S6*B*) or under continuous red light (Fig. S6*C*). *lnk2* mutants also had longer hypocotyls than WT plants in red light (Fig. S6*C*), whereas the differences in hypocotyl length were not statistically significant under most other light conditions (Fig. 2*A*; Fig. S6). The *lnk1;lnk2* double mutant had significantly longer hypocotyls than either single mutant or WT seedlings under continuous white light conditions, and the phenotype was stronger under red or white light than under blue light (Fig. 2*A*; Fig. S6). Taken together, these results indicate that LNK1 and LNK2 mediate light inhibition of hypocotyl elongation, in particular that triggered by the phytochrome family of red/far-red light photoreceptors.

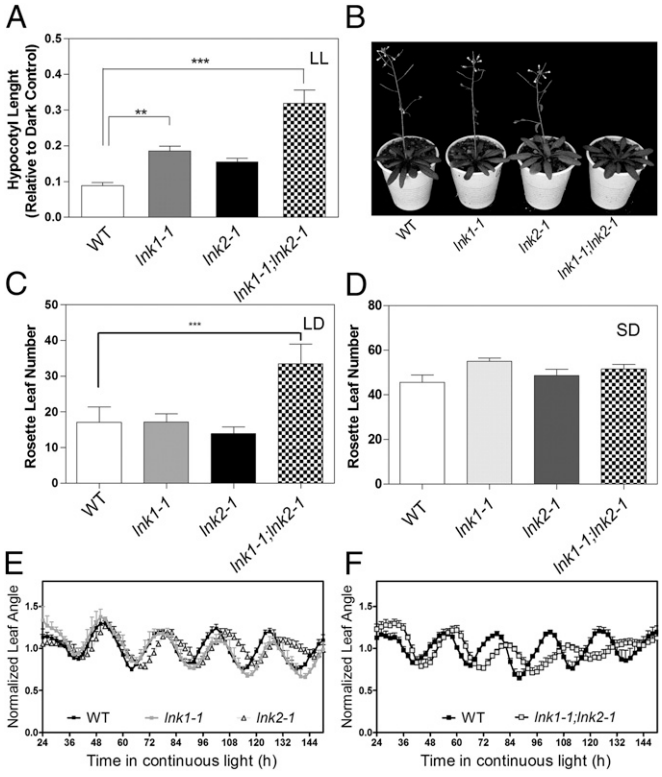

Fig. 2. Physiological characterization of *LNK1* and *LNK2*. (*A*) Hypocotyl length of WT, *lnk1*, *lnk2,* and *lnk1;lnk2* mutant seedlings grown under continuous white light (LL) ($n = 6$ replicates of 10 seedlings each). (*B*) *LNK1* and *LNK2* control the floral transition in plants grown under LD (16-h light/8-h dark) conditions. (*C* and *D*) Flowering time measured as the number of rosette leaves at bolting in LD (*C*) and SD (*D*) conditions (8-h light/16-h dark). ANOVA followed by a Tukey´s multiple comparison test was used to evaluate the statistical significance of differences observed between genotypes. Error bars indicate +SEM (***$P <$ 0.001, *$P < 0.05$). (*E* and *F*) Circadian rhythms of leaf movement in continuous light ($n = 7$). Plants were grown under LD cycles and then transferred to constant light and temperature conditions. Error bars indicate +SEM. Open and hatched boxes indicate subjective day and subjective night, respectively.

Another physiological process that depends on the interactions between light signaling and the circadian clock is photoperiod-dependent flowering (25). *lnk1;lnk2* double mutant flowered later than WT plants or *lnk1* or *lnk2* single mutants under long days (LD; 16-h light/8-h dark; Fig. 2 *B* and *C*). Under short days (SD; 8-h light/16-h dark), no delay in flowering was observed (Fig. 2*D*), confirming that LNK1 and LNK2 are indeed only required for long day–dependent acceleration of flowering rather than the transition to flowering per se.

To observe circadian behavior directly, we monitored the circadian rhythm of leaf movement in WT plants and *lnk1*, *lnk2*, and *lnk1;lnk2* mutants by time lapse photography. Leaf movement of *lnk2* mutants had a longer circadian period than WT or single *lnk1* mutant plants (Fig. 2*E*; Fig. S6*F*), and the *lnk1;lnk2* double mutant was even more strongly affected (Fig. 2*F*; Fig. S6*F*). Similar photomorphogenic and circadian phenotypes were observed in additional mutant alleles of *LNK1* and *LNK2* (Fig. S6), confirming that these two genes play important and partially redundant roles controlling light- and clock-regulated processes in *Arabidopsis*.

LNK1 and LNK2 Activate Clock-Controlled Genes with Afternoon Peak. LNK proteins lack known functional domains, but *LNK1:YFP* localized mostly to the nucleus in *Arabidopsis thaliana* hypocotyl cells, suggesting a role in the regulation of gene expression (Fig. 3*A*). To identify genes controlled by LNK1 and LNK2, we compared the transcriptome of WT and *lnk1;lnk2* mutant plants using RNA sequencing (RNA-seq). In plants grown under constant light and temperature, we found 806 genes differentially expressed using a false discover rate (FDR)-adjusted $P < 0.01$ as a cutoff (Dataset S3*A*). Genes down-regulated in *lnk1;lnk2* mutants were significantly enriched for genes that peak in LD at Zeitgeber time 10 (ZT10), i.e., 10 h after lights on. Up-regulated genes were slightly enriched for genes that peak late at night (Fig. 3*B*).

To learn more about LNK1/LNK2 target genes, we used RNA-seq to characterize the daily transcriptome of LD-grown WT and *lnk1;lnk2* mutant plants. Using stringent criteria aimed at identifying genes with altered overall mRNA levels, and not simply changed temporal patterns of expression, we identified 387 genes that differed between WT and *lnk1;lnk2* mutant plants (Dataset S3*B*). A cluster analysis revealed that most of the genes down-regulated in *lnk1;lnk2* mutant oscillated in WT plants with peak expression in the afternoon or early night (Fig. S7), with the largest cluster peaking at ZT10 (Fig. 3*C*), providing independent support for the initial phase enrichment analysis, which had suggested that LNK1/LNK2 activity is maximal in the afternoon (Fig. 3*B*).

To identify genes likely responsible for the phenotypic defects in *lnk1;lnk2* mutants, we focused our analysis on the 101 down-regulated and the 31 up-regulated genes in both RNA-seq data sets (Dataset S3*C*). Down-regulated genes included two core-clock genes, *PRR5* (Fig. 3*D*) and *EARLY FLOWERING 4* (*ELF4*; Fig. 3*E*), which were present in the cluster of genes with peak expression in WT plants at ZT10 (Fig. 3*C*) and might be primary targets of LNK1/LNK2 activity. Other clock and light signaling genes were also misregulated in *lnk1;lnk2* mutants (Dataset S3*C*). However, these genes were affected more subtly, suggesting that they might be secondary targets of LNK1/LNK2 activity. Down-regulated genes also included the flowering time genes *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF1*; Fig. 3*F*), which was also present in the cluster of genes with peak

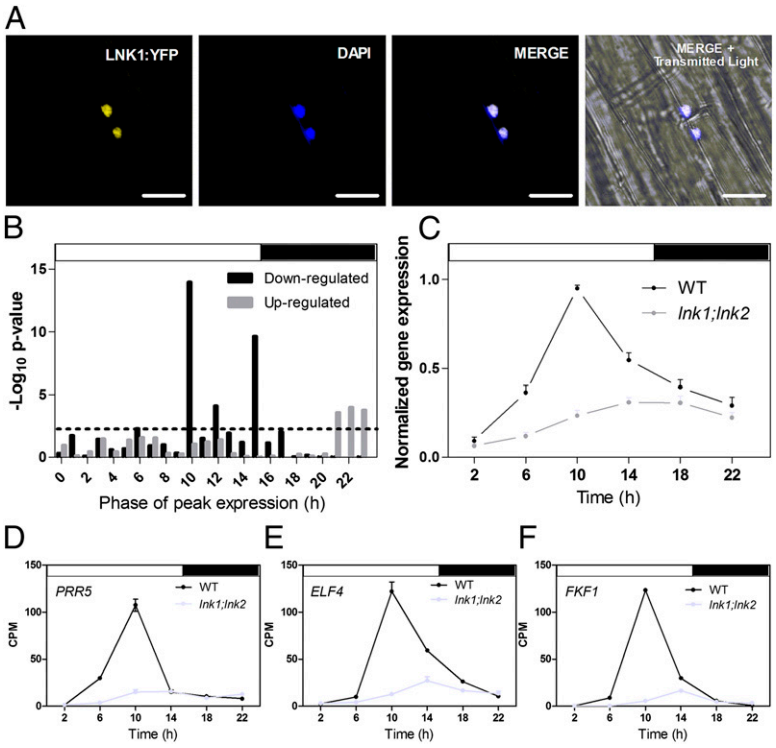

Fig. 3. LNK1, a nuclear protein, positively regulates expression of circadian genes with an afternoon phase. (*A*) LNK:YFP detection by confocal microscopy in the hypocotyl of seedlings expressing *35S:LNK:YFP* in a WT background is shown on the left (first panel). Fluorescence following staining with DAPI is shown in blue (second panel). The merged image (white) and the image with the transmitted light channel are also shown (third and fourth panels, respectively). (Scale bar, 20 μm.) (*B*) Phase enrichment of circadian-regulated genes whose expression was down- or up-regulated in *lnk1;lnk2* mutant compared with WT plants, according to RNA-seq data of plants grown under continuous light conditions. The phase overrepresentation analysis was conducted with Phaser (http://phaser.mocklerlab.org/) and was based on the phases of gene expression estimated from data obtained using WT plants grown under LD conditions (23). Dashed line corresponds to $P = 0.01$. (*C*) Average normalized expression of 36 genes from the cluster with the largest number of genes whose expression was altered in *lnk1:lnk2* mutants compared with WT plants grown under LD conditions. Normalized expression of *PRR5* (*D*), *ELF4* (*E*), and *FKF1* (*F*), three genes present in the cluster shown in *C*. (*C–F*) Plants grown under LD cycles were sampled every 4 h, starting 2 h after lights on. $n = 3$, Error bars indicate + SEM.

expression at ZT10 (Fig. 3*C*), as well as *FLOWERING LOCUS T* (*FT*) and *SUPPRESOR OF CONSTANS OVEXPRESSION 1* (*SOC1*) (Fig. S8). All three genes are positive regulators of flowering time, with *FKF1* acting upstream of *FT* and *SOC1* (26). Therefore, the late flowering of *lnk1;lnk2* mutants under LD is likely due, at least in part, to reduced *FKF1* expression, which in turn leads to reduced *FT* and *SOC1* mRNA levels (Fig. S8). *FT* expression is controlled by the transcription factor CONSTANS (CO), whose transcript and protein levels are independently regulated by FKF1 (26, 27). *CO* transcript levels were only slightly reduced in *lnk1;lnk2* mutants throughout the afternoon of a long day (Fig. S8), suggesting that the strong down-regulation of *FT* mRNA levels in the *lnk1;lnk2* mutants might result from an effect of FKF1 on CO protein (27).

***PRR5* Expression Is Severely Affected in *lnk1;lnk2* Mutants Under Free-Running Conditions.** To investigate the effect of LNK1 and LNK2 on the central clock in more detail, we analyzed the expression of clock components in plants that had been entrained under 12-h light/12-h dark cycles at 22 °C and were then transferred to constant light and temperature (i.e., free-running) conditions. The plant circadian clock is based on interlocking transcriptional feedback loops in which the morning clock factors CCA1 and LATE ELONGATED HYPOCOTYL (LHY) repress the expression of evening clock genes such as *TOC1/PRR1* (28). In addition, CCA1 and LHY also promote the expression of *PRR9* and *PRR7* (29), which, sequentially with *PRR5* and *TOC1/PRR1*, repress *CCA1* and *LHY* expression throughout the remaining of the day and early night (30–32).

We observed a substantial delay in the phase of *CCA1*, *LHY*, *PRR9,* and *PRR7* expression during the second day in continuous light. The delay increased to 8 h on the third day, consistent with a lengthening of circadian period by ∼2.5 h in the *lnk1;lnk2* mutant compared with WT plants (Fig. 4 *A–D*; Fig. S6*F*). Despite the strong effect of LNK1 and LNK2 on the period and/or phase of circadian oscillations, the overall mRNA levels of morning and early afternoon clock components were largely unaffected in *lnk1;lnk2* mutants. In contrast, significant down-regulation coupled to a much longer delay in the phase of expression, i.e., close to 12 h on the third day, was observed for *PRR5*, which is normally expressed in the afternoon (Fig. 4*E*). A similar phase delay, but lacking differences in the overall mRNA levels, was observed for the *TOC1/PRR1* gene that is expressed slightly after *PRR5* (Fig. 4*F*). A strong delay in the timing of *TOC1* expression, coupled with a slight reduction in overall levels, was observed for this clock gene under LD conditions (Fig. S9). Taken together, these results suggest that LNK1 and LNK2 act initially as transcriptional activators, controlling the levels and timing of expression of a subset of genes with peak expression in the afternoon, such as *PRR5* (Figs. 3*D* and 4*E*), *ELF4* (Fig. 3*E*), *TOC1* (Fig. S9), and *FKF1* (Fig. 3*F*), which later affect the rhythmic expression of other core-clock and clock-output genes.

***LNK1* and *LNK2* Are Repressed by Members of the TOC1/PRR1 Family of Clock Genes.** Many clock-regulated genes with peak expression in the morning are repressed throughout the day and during the early night by members of the TOC1/PRR1 family of clock proteins. To determine whether the LNKs were regulated by members of this protein family, we reexamined data describing TOC1/PRR1 and PRR5 binding sites in the *Arabidopsis* genome using ChIP followed by sequencing (ChIP-seq) (31, 33). Indeed, we found that the regulatory region of *LNK3* was directly bound by TOC1/PRR1 (31). ChIP followed by qPCR not only confirmed this, but also revealed that TOC1/PRR1 binds directly to the regulatory regions of *LNK1*, *LNK2*, and *LNK4* (Fig. 5*A*). Furthermore, PRR5, PRR7, and PRR9 were also found to bind directly to the regulatory regions of *LNK1–4* (33).

To evaluate the functional consequence of the binding of these factors to *LNK1–4* promoters, we compared the expression patterns of *LNK1* and *LNK2* in WT, *toc1,* or *prr9;prr7* mutant plants, entrained under light/dark cycles and then transferred to constant light conditions. Not only did we observe progressively larger delays in the phase of the circadian oscillations of *LNK1* and *LNK2*, but their mRNA levels were increased in the *prr9;prr7* double mutant at the trough of the circadian oscillations (Fig. 5 *B* and *C*). A larger overall increase in *LNK1* and *LNK2* mRNA levels,

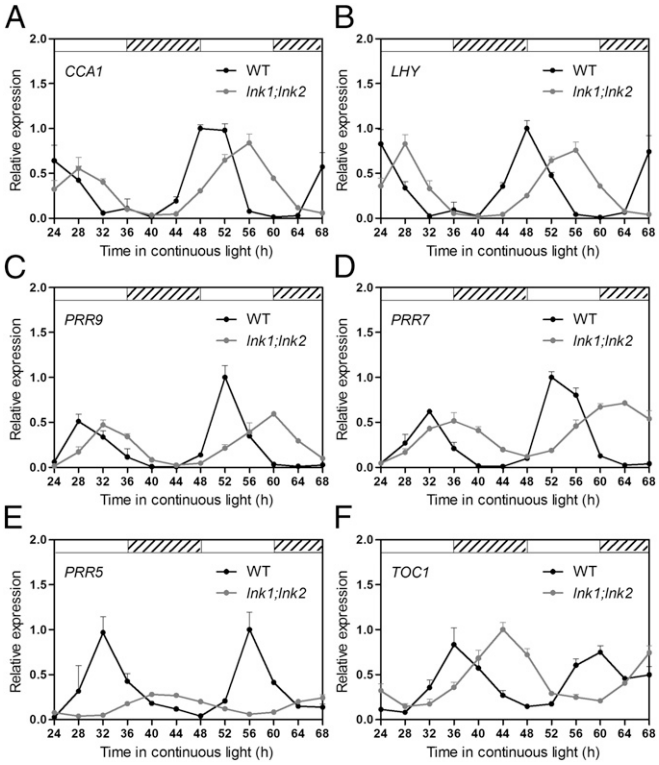

Fig. 4. *LNK1* and *LNK2* are necessary for the proper function of the circadian clock. *CCA1* (*A*), *LHY* (*B*), *PRR9* (*C*), *PRR7* (*D*), *PRR5* (*E*), and *TOC1* (*F*) mRNA expression measured by qRT-PCR in plants grown under 12-h light/12-h dark cycles and then transferred to continuous light. Values are expressed relative to *PP2A* and normalized to the maximum value of each gene. Data represent average +SEM ($n = 4$). Open and hatched boxes indicate subjective day and subjective night periods, respectively.

coupled to progressive phase advances, was also observed in the short period mutant *toc1* over the entire time course (Fig. 5 *D* and *E*), indicating that TOC1 is a direct repressor of these genes.

Discussion

Light and the circadian clock interact to regulate many biological processes in plants, such as flowering time (25) and stem growth (18, 19). In addition, this interaction is also required for robust functioning of the circadian clock itself (15, 20). Our genomewide analysis revealed that these physiological interactions are mirrored by global interactions at the transcriptional level. In particular, we found that 70% of light-induced genes responded more strongly to a light pulse during the subjective day than during the night, likely optimizing the energy spent on light-dependent biological processes that have maximal activity at midday, when light intensity is at its peak under natural conditions. At the same time, a light stimulus during the night preferentially promoted the expression of certain key clock components, consistent with the general observation that light present at the beginning or end of the photoperiod adjusts the circadian clock to seasonal changes in day length (2, 21).

The characterization of genes that are preferentially induced by light at night and that are also rhythmic across multiple conditions led to the identification of *LNK* genes, a partially redundant family of plant-specific genes that control photomorphogenic and photoperiodic responses, as well as circadian rhythms. *LNK1* and *LNK2* are regulated by the phytochrome photoreceptors and predominantly affect photomorphogenic responses to red light, pointing to an important role in phytochrome signaling. Additionally, *LNKs* are expressed rhythmically with peak expression in the morning or at noon, likely due to their repression by members of the TOC1/PRR1 family of core clock regulators during the afternoon and early night. Thus, LNK1 and LNK2 link phytochrome and circadian signaling to regulate many physiological processes, including time keeping by the clock itself.

A comparison of *LNK* genes with other light-induced clock genes or regulators is informative. Like *LNK* genes, *CCA1* and *LHY* are light-induced genes whose mRNAs reach peak levels in the early morning (9, 34). Mutations in *CCA1* and *LHY*, however, shorten the period of circadian rhythms, whereas mutations in *LNK1* and *LNK2* lengthen it (6). *GI* is a light-induced clock regulator, and loss-of-function mutations in this gene lengthen circadian period but, in contrast to *LNK1* and *LNK2*, *GI* is expressed with peak levels in the late afternoon (35, 36). Finally, *PRR9* and *PRR7* are similar to *LNK1* and *LNK2* in that they are expressed during the morning and early afternoon, are induced by light, and decrease period length and promote flowering (29). Different from *LNK2* and *LNK2*, which at least under constant light do not seem to be required for normal *CCA1* and *LHY* expression (Fig. 4), *PRR9* and *PRR7* are repressors of *CCA1* and *LHY* (29). Thus, LNK1 and LNK2 act differently from previously described light-induced clock genes or regulators.

LNK1 and LNK2 are plant-specific proteins without recognizable functional domains. This is reminiscent of the clock

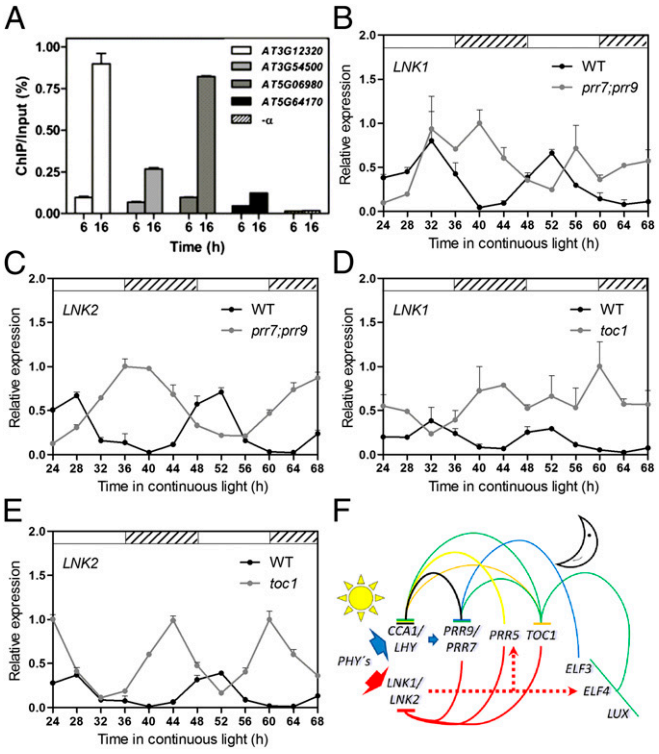

Fig. 5. *LNK1* and *LNK2* are repressed by the TOC1/PRR1 family of circadian clock components. (*A*) TOC1 binds to *LNK1–4* gene promoters. ChIP-qPCR assays were conducted using TOC1. Minigene (TMG) seedlings grown under 12-h light/12-h dark cycles. Samples were collected at ZT 6 and ZT16 in the light and dark, respectively. (*B–E*) *LNK1* (*B* and *D*) and *LNK2* (*C* and *E*) expression measured by qRT-PCR in continuous light relative to *PP2A* ($n = 4$). Plants were grown under 12-h light/12-h dark cycles and then transferred to continuous light. Error bars indicate +SEM. Open and hatched boxes indicate subjective day and subjective night, respectively. (*F*) Model showing the proposed function of LNK1 and LNK2 in the circadian clock. Light regulates *LNK1* and *LNK2* expression in the morning, which then act to promote, directly or indirectly (dashed line), the expression of a subset of afternoon genes, including the core clock genes *PRR5* and *ELF4*. During the afternoon and early evening, PRR9, PRR7, PRR5, and TOC1 bind to the LNK promoters blocking their expression.

components ELF3 and ELF4, which only very recently were shown to participate in an evening phased protein complex that represses the expression of a subset of morning genes, such as *PRR9* (19, 37–39). The precise mechanism through which LNK1 and LNK2 affect the pace of the clock is uncertain. They activate the expression of afternoon/early evening genes, including *PRR5* and *ELF4*, but the long period phenotype is unlikely to be simply the result of reduced expression of these two genes. If that was the case, *lnk1;lnk2* mutants should be either short period or arrhythmic, such as *prr5* or *elf4* mutants, respectively. Thus, the long period phenotype may result from delayed activation of afternoon/early evening genes rather than simply from, or in addition to, reduced levels of these genes. In summary, our work supports a model in which light perceived through phytochromes activates the expression of the *LNKs*, as well as that of the *CCA1* and *LHY* (9), in the early morning. CCA1 and LHY then promote the expression of *PRR9* and *PRR7* (29), whereas LNK1 and LNK2 act later during the day to activate clock genes with peak expression in the afternoon, such as *PRR5* and *ELF4*. Simultaneously, members of the TOC1/PRR family repress these morning genes throughout the afternoon and beginning of the night (30–32, 40). Finally, the progressive reduction in TOC1/PRR levels leave *CCA1*, *LHY,* and the *LNK* genes poised to respond again to light signals that reset the clock every morning (Fig. 5*F*).

Materials and Methods

Plant Material. All of the *Arabidopsis* lines used in this study were Columbia ecotype. *lnk1-1* (SALK_024353), *lnk1-2* (SALK_063322), *lnk1-3* (GK_044A09), *lnk2-1* (GK_484F07), *lnk2-2* (SALK_116103), and *lnk2-3* (SALK_141609) mutants were obtained from the *Arabidopsis* Biological Research Center (ABRC) and the Gabi Kat T-DNA insertion collections. The *lnk1;lnk2* double mutant was obtained by crossing the simple mutants *lnk1-1* and *lnk2-1*. The clock and photoreceptor mutants used in this study were *prr7-3;prr9-1*, *toc1-101*, *phyA-211;phyB-9,* and *cry1-b104;cry2-1*.

Growth Conditions. For flowering time experiments, the plants were grown on soil at 22 °C under long days (LD; 16-h light/8-h dark cycles; 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white light), short day (SD; 16-h light/8-h dark cycles; 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white light), or continuous light (LL; 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white light), depending on the experiment.

Physiological Measurements. Detailed information is in *SI Materials and Methods*.

Subcellular Localization of LNK1. Detailed information is in *SI Materials and Methods*.

qRT-PCR, Microarray, and RNA-Seq Analysis. Detailed information is in *SI Materials and Methods*.

ChIP Analysis. Detailed information is in *SI Materials and Methods*.

Protein Sequence Alignment and Phylogenetic Analysis. Detailed information is in *SI Materials and Methods*.

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