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* (*ELF3*) (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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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE43865 and GSE46621).

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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. 1A). 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. 1B; 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 S1A), and only 65 genes showed a stronger response during the night (Dataset S1B). Thus, almost 70% of light-induced genes behaved similarly to what had been reported for CABI1 (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). 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 S1C), and 78 showed the opposite response (Dataset S1D). The group more strongly repressed by light during the subjective day was mostly enriched in genes involved in amino acid catabolism (Dataset S2B), whereas those more responsive to light during the night were associated with hormonal regulation, among other processes (Dataset S2C).

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 *cry1cry2* 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.

**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 S2D). 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 × 10−12, hypergeometric distribution; Fig. 1C). 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 light night–inducible and clock-regulated genes 1–4 (Fig. 1D).

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 S1B), 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. S6A). In contrast, lnk1 mutants had longer hypocotyls than WT plants under continuous white light (Fig. 2A; Fig. S6B) or under continuous red light (Fig. S6C). lnk2 mutants also had longer hypocotyls than WT plants in red light (Fig. S6C), whereas the differences in hypocotyl length were not statistically significant under most other light conditions (Fig. 2A; 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. 2A; 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.

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. 2B and C). Under short days (SD; 8-h light/16-h dark), no delay in flowering was observed (Fig. 2D), 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. 2E; Fig. S6F), and the lnk1;lnk2 double mutant was even more strongly affected (Fig. 2F; Fig. S6F). 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. 3A). 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 S3A). 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. 3B).

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 S3B). 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. 3C), providing independent support for the initial phase enrichment analysis, which had suggested that LNK1/LNK2 activity is maximal in the afternoon (Fig. 3B).

To identify genes likely responsible for the photophytropic 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 S3C). Down-regulated genes included two core-clock genes, PRR5 (Fig. 3D) and EARLY FLOWERING 4 (ELF4; Fig. 3E), which were present in the cluster of genes with peak expression in WT plants at ZT10 (Fig. 3C) and might be primary targets of LNK1/LNK2 activity. Other clock and light signaling genes were also misregulated in lnk1;lnk2 mutants (Dataset S3C). 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. 3F), which was also present in the cluster of genes with peak
expression at ZT10 (Fig. 3C), as well as FLOWERING LOCUS T (FT) and SUPPRESSOR OF CONSTANS OVEREXPRESSION 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/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 HYOCOTYL (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. S6F). 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. 4E). 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. 4F). 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. 3D and 4E), ELF4 (Fig. 3E), TOC1 (Fig. S9), and FKF1 (Fig. 3F), 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. 5A–D). 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, tocl1, 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. 5B and C). A larger overall increase in LNK1 and LNK2 mRNA levels,
coupled to progressive phase advances, was also observed in the short period mutant toci 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 photophenotypic 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. 4.](Image)

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.

![Fig. 5.](Image)

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, 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/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 the LNKS act later during the day to activate clock genes with peak expression in the afternoon, such as PRR5 and ELF4. LNK2 supports a model in which light perceived through phytochromes add to, reduced levels of these genes. In summary, our work suggests that the long period phenotype may result from delayed activation of PRR5 and ELF4.

Materials and Methods

Plant Material. All of the Arabidopsis lines used in this study were Columbia ecotype. Ink-1-1 (SALK_024353), Ink-1-2 (SALK_063322), Ink-3 (GK_044A09), Ink2-1 (GK_484F07), Ink2-2 (SALK_116103), and Ink2-3 (SALK_141609) mutants were obtained from the Arabidopsis Biological Research Center (ABRC) and the Gabi Kat T-DNA insertion collections. The Ink1;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, phyb-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 μmol m−2 s−1 of white light), short day (SD; 16 h light/8 h dark cycles; 140 μmol m−2 s−1 of white light), or continuous light (LL; 50 μmol m−2 s−1 of white light), depending on the experiment.

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

Subcellular Localization of LNKS. 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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Supporting Information

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SI Materials and Methods

**Mutant Allele Confirmation of Night Light–Inducible and Clock-Regulated Genes.** Plants were grown in soil for 3 wk. Samples from four plants per genotype were collected to reduce biological variation. RNA was obtained using TRIzol reagent (Invitrogen). One microgram of RNA was treated with RQ1 RNase-Free DNase (Promega) and subjected to retrotranscription with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen) and oligo-dT according to manufacturer’s instructions. Transcript abundance of night light–inducible and clock-regulated genes (LNK genes) was determined by PCR to confirm the null or reduced expression in each line. ACT2 was used as an expression control. Primers used for LNK cDNA expression analysis were as follows: lnk1-1, forward: AGGTGACTTAGGGTGGTTCTCTTC, reverse: CTCGGCAAAACTTGTGCTTCC; lnk1-2, forward: TGGAGGTCTAGTGGAAGAACTC, reverse: TTGTTCCCCGTATCAGGAGAAAAC; lnk1-3, forward: AGCTTACAAGGTTCCTCACGTGTGATT, reverse: TGGCAGGCTCGGGCTCCCTTCTC; lnk2-1/lnk2-2/lnk2-3, forward: GGTGAGAGGGTTGTTGAAGCATC, reverse: AAAGGAGCGTACGAGGATTGTTC.

**Physiological Measurements.** Flowering time was estimated by counting the number of rosette leaves at the time of bolting. For hypocotyl length measurements, WT, lnk1, lnk2, and lnk1;lnk2 seedlings were grown on 0.8% agar under complete darkness, continuous white light (LL), short day (5-h light/16-h dark) photoperiods, continuous red (100 μmol·m⁻²·s⁻¹) or continuous blue light (10 μmol·m⁻²·s⁻¹), and the final length of the hypocotyls was measured after 4 d. Light effects on hypocotyl elongation were calculated normalizing hypocotyl length under each light regime relative to hypocotyl length of the same genotypes under constant dark conditions. For leaf movement analysis, plants were grown under 16-h light/8-h dark cycles and transferred to continuous 20 μmol·m⁻²·s⁻¹ fluorescent light at 22 °C, and the position of the first pair of leaves was recorded every 2 h for 6 d using digital cameras and determined using Image J software.

**Subcellular Localization of LNK1.** Subcellular localization of LNK1 was determined by analyzing T1 transgenic plants of the WT Columbia accession transformed with the 35S::LNK1:YFP construct. Hypocotyl tissue from 10-d-old plants was analyzed. Five independent transgenic lines were observed showing similar results. Transgenic plants were obtained using the floral dip transformation method (1). The 35S::LNK1::YFP construct was assembled as follows. The AT5G64170.2 coding sequence, obtained from the Arabidopsis Information Resource (TAIR10), was synthesized de novo by GeneScript Corporation and then introduced into the pearly gate101 destination vector (2) using Gateway technology (Invitrogen). Imaging was performed using a LSM 510 META confocal microscope equipped with a 405-nm diode and an argon ion (488 nm) excitation laser system and a 40×, NA 1.3 objective. Images were processed with the LSM image browser software.

**Quantitative RT-PCR.** For time course analysis, 15-d-old plants were grown under 12-h light/12-h dark cycles at 22 °C and then transferred for 3 d to continuous white light at 22 °C. Samples were collected every 4 h for 2 d, starting 24 h after transfer to constant conditions. Total RNA was obtained from these samples using TRIzol reagent (Invitrogen). One microgram of RNA was treated with RQ1 RNase-Free DNase (Promega) and subjected to retrotranscription with M-MLV (Invitrogen) and oligo-dT according to the manufacturer’s instructions. Synthesized cDNAs were amplified with FastStart Universal SYBR Green Master (Roche) using the Mx3000P Real Time PCR System (Agilent Technologies) cycler. The PP2A (AT5G13320) transcript was used as a housekeeping gene. Quantitative RT-PCR (qRT-PCR) quantification was conducted using the standard curve method as described in the Methods and Applications Guide from Agilent Technologies. Primer sequences and PCR conditions are available on request.

**Phylogenetic Analysis.** Homologs of Arabidopsis thaliana LNK1 (splice variant 2) were identified using TBLASTN (www.phytozome.net/). Protein sequences were aligned using the Clustal Omega program. A maximum likelihood phylogenetic tree was built using SeaView Version 4 (3), with 1,000 bootstrap replicates.

**Microarray Analysis.** Total RNA was extracted from the entire aerial structure of 15-d-old WT plants grown under 12-h light/12-h dark cycles at 22 °C. Triplicate samples were collected after a 1-h white light treatment (70 μmol·m⁻²·s⁻¹) in the middle of the night or subjective day, and control samples kept in darkness were collected at the same time. Each replicate consisted of 10–12 plants to reduce biological variation. Total RNA was processed and hybridized to Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays, according to the manufacturer’s instructions. Data were analyzed using MAS5, and ANOVA was used to identify differentially expressed genes [q value < 0.0005 (4); fold change ≥2]. Genes were then classified into different groups according to the relative effect of the light pulse given during the night compared with the effect of the same treatment given during the subjective day (Dataset S1). These groups include those in which the effect of light, i.e., induction or repression of gene expression, was at least twice as large during the subjective day than at night, those that showed a response that was at least twice as large during the night compared with subjective day, and finally, those in which the difference between the effect during the subjective day and night was not larger than twofold (Dataset S1). Microarray data have been deposited in Gene Expression Omnibus (GEO; accession nos. GSE46741 for the superseries and GSE46621 for the subseries corresponding to the microarray data set).

**Functional Category Enrichment Analysis.** Functional categories associated with specific groups of light-regulated genes were identified using the BioMaps tool from the virtual plant software (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb). This tool allowed us to determine which functional categories were statistically overrepresented in particular lists of genes compared with the entire genome (5).
(Illumina). Briefly, 3 μg of total RNA was polyA-purified and fragmented, and first-strand cDNA synthesized by reverse transcriptase (SuperScript II; Invitrogen) and random hexamers. This was followed by RNA degradation and second-strand cDNA synthesis. End repair process and addition of a single A nucleotide to the 3′ ends allowed ligation of multiple indexing adapters. Then, an enrichment step of 12 cycles of PCR was performed. Library validation included size and purity assessment with the Agilent 2100 Bioanalyzer and the Agilent DNA 1000 kit (Agilent Technologies). Samples were pooled to create 17 multiplexed DNA libraries, which were single-end sequenced with an Illumina Genome Analyzer II kit on the Illumina GAIX platform, providing 100-bp single-end reads.

Processing of RNA Sequencing Reads. RNA sequencing (RNA-seq) reads were analyzed using Illumina Pipeline version 1.3. Reads were quality-filtered using the standard Illumina process and demultiplexed with two allowed barcode mismatches. Sequence files were generated in FASTQ format. Table S1 provides a summary table of main read count statistics. Sequence data have been deposited in GEO (accession no. GSE43865). The TopHat suite (6) was used to map reads to the A. thaliana TAIR10 reference genome. Along with the prebuilt A. thaliana index, the reference genome was downloaded from ENSEMBL (December 2012). Default values for TopHat parameters were used with the exception of maximum intron length parameter, which was set to a value of 5,000 nt following estimated values reported in ref. 7.

RNA-seq Data Processing and Differential Expression Analysis. Several packages from the Bioconductor library (version 2.11) of the R (version 2.15) statistical analysis framework were used to quantify gene differential expression signals. Default values were used unless explicitly stated otherwise. Package easyRNASeq (8) was used to generate read count tables at the gene level from Binary Alignment Map (BAM) files. A nonspecific prefiltering step was then conducted to filter out genes with less than two counts per million reads present along the whole set of samples, resulting in 21,143 (22,628) of 33,602 genes that were considered for further analysis in the WT vs. lnk1;lnk2 (time course) experiment. The subsequent normalization and statistical analysis of read count data were performed using the package edgeR (version 3.04) following general guidelines provided in refs. 9 and 10. First, differences in RNA composition for each library were taken into account through a normalization step using the trimmed mean of M values (TMM) methodology. Then, estimates of the dispersion parameter for each transcript were obtained in a two-step procedure using the functionality implemented in estimateGLMTrendedDisp and estimateGLMATagwiseDisp functions (10). To assess differential expression, a negative binomial generalized log-linear model was fitted to each gene read counts using the glmFit function. Finally we used glmLRT to conduct gene-wise statistical tests for the coefficient contrasts of interest.

Following this analysis pipeline, we found 806 genes differentially expressed between lnk1;lnk2 and WT Col conditions [Benjamini-Hochberg false discover rate (FDR)-adjusted P < 0.05]. For the more complex time course experiment, statistical significance tests were performed for mean differences between WT and lnk1;lnk2 mutant time courses, along with genotypetime interaction contrasts for time points 6, 10, 14, 18, and 22 h. We then focus our attention on genes that simultaneously fulfilled the following two conditions: transcripts should present large (fold change >1.5) and significant (Benjamini-Hochberg FDR-adjusted P < 0.0001) changes in lnk1;lnk2 vs. WT mean expression level along the time course and at least one significant (Benjamini-Hochberg FDR-adjusted P < 0.0001) genotype-time interaction contrast. In this way, a subset of 387 transcripts were identified and considered for follow-up analysis.

Expression Profile Clustering. To analyze patterns of coordinated gene expression behavior, read counts were log-transformed after a minimal offset (offset level = 1e−6) was added to avoid zero count values. Then, for each transcript, an extended expression profile was defined concatenating the WT and lnk1;lnk2 time course profiles. A correlation-based similarity measure between extended expression profiles Ti and Tj was considered, and the distance metric dij = 0.5[1 – cor(Ti, Tj)] was used to perform a hierarchical clustering (complete linkage) of gene profiles. Finally, clusters of coordinated expression were obtained from the dendrogram structure with the aid of the dynamicTreeCut (11) package.

ChIP Analysis. ChIP assays were performed essentially as described in Huang et al. (12).

**Fig. S1.** Genomewide analysis of sucrose and CO\(_2\) effects on gene expression of light-regulated genes. Comparative analysis of the effect on gene expression of a light pulse given during subjective day time (x axis) vs. the effect of sucrose added to plants undergoing starvation (y axis) or the effect of enhanced photosynthetic activity resulting from increased CO\(_2\) levels. (A and B) Light-induced genes. (C and D) Light-repressed genes. Data for changes in gene expression resulting from added sucrose or enhanced photosynthetic activity were obtained from Osuna et al. (1). Data for light effects during the subjective day correspond to results described in Dataset S1.


**Fig. S2.** Light effects on gene expression during the subjective day in plant photoreceptor mutants and comparison with the effect of sucrose and photosynthesis. Changes in gene expression measured by qRT-PCR in two light-induced (A–D) and two light-repressed (E–H) genes shown as fold change. (A, C, E, and G) Effect of a light pulse given in the middle of the subjective day on AT5G64170 (LNK1) (A), AT1G22770 (GI) (C), AT2G33810 (SPL3) (E), and AT4g27260 (G) expression in WT, phyA;phyB, and cry1;cry2 mutant plants. Data represent average ± SEM (n = 3). (B, D, F, and H) Effect of sucrose or CO\(_2\) addition on the expression of the same genes indicated above. Data for changes in gene expression resulting from added sucrose or enhanced photosynthetic activity were obtained from Osuna et al. (1).

Fig. S3. Sequence alignment of LNK homologs in Arabidopsis thaliana. The length of the sequence aligned is shown, and degree of similarity between amino acids is highlighted (darker blue indicates higher similarity).
Fig. S4. Cladogram displaying LNK1 homologs in a broad range of species from embryophyta group. Arabidopsis thaliana LNK1 has three paralogs. Where multiple homologs were identified within a single species, the annotated gene model code is provided. Rc, Ricinus communis; Pp, Physcomitrella patens; Solyc, Solanum lycopersicum; Caruv, Capsella rubella; Os, Oryza sativa; Cs, Cucumis sativus; Medtr, Medicago truncatula; Sb, Sorghum bicolor. Percentage bootstrap values are presented for each node.
Fig. S5. LNK1 and LNK2 expression in different mutant backgrounds. (A) Scheme of LNK1 and LNK2 showing the site of T-DNA insertions in the different mutant alleles. (B) All mutant alleles have strongly reduced expression of the full-length mRNA, evaluated using primers flanking the T-DNA insertion. Plants were grown in soil for 3 wk in continuous light conditions. Samples harvested were processed until cDNA synthesis. Transcript presence was determined by PCR.
Fig. S6. Physiological characterization of different Ink1 and Ink2 mutant lines. (A–D) Hypocotyl length of plants grown for 4 d under different light conditions. (A) DD, continuous darkness. (B) LL, continuous white light. (C) Rc, continuous red light. (D) Bc, continuous blue light. Hypocotyl length under different light conditions is expressed relative to the hypocotyl length of each genotype under continuous darkness. (E) Flowering time measured as the number of rosette leaves at bolting in LL. (F) Period length differences between mutant and WT plants in the circadian rhythm of leaf movement (period length of WT plants = 24.2 ± 0.16; n = 7). Period length was calculated by BRASS 3.0 software. ANOVA followed by a Tukeys multiple comparison test was used to evaluate statistical significance of the difference with WT plants. Error bars indicate ±SEM (***P < 0.001, **P < 0.01, *P < 0.05).
Fig. S7. Gene clusters identified by RNA-seq analysis. (A–I) Clusters of genes with similar expression patterns detected using a correlation-based distance metric and a hierarchical clustering procedure followed by a hybrid adaptive dendrogram cut step. Data sets represent the average of normalized expression level for all genes within each cluster. Number of genes in each cluster is indicated between parentheses. Plants were grown and harvested in 16-h light/8-h dark cycles. Clusters obtained for genes down-regulated (A–E) or up-regulated (G–I) in \textit{lnk1;lnk2} mutant. (F) Cluster formed by genes with a significant alteration in the temporal pattern of expression but without large differences in expression levels between WT and \textit{lnk1;lnk2}.
Fig. S8. Expression levels of flowering time genes in WT and Ink1;Ink2 mutant plants. Data were from the RNA-seq experiment. cpm, counts per million.
Fig. S9. TOC1 expression under LD conditions in WT and lnk1;lnk2 mutant plants. Data were from the RNA-seq experiment. cpm, counts per million.

Table S1. Read counts summary stats

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Minimum library size</th>
<th>Maximum library size</th>
<th>Median library size</th>
<th>Correlation between replicates</th>
<th>Correlation between conditions</th>
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<tbody>
<tr>
<td>LL</td>
<td>14,074,859</td>
<td>18,297,931</td>
<td>15,855,443</td>
<td>0.9893, 0.9986</td>
<td>0.9677, 0.9986</td>
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<tr>
<td>Time course</td>
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<td>13,408,078</td>
<td>11,350,789.67</td>
<td>0.8758, 0.9995</td>
<td>0.827, 0.9995</td>
</tr>
</tbody>
</table>

Dataset S1. Genes differentially regulated by light during the middle of the subjective day or night

Dataset S1 (XLSX)

Dataset S2. GO enrichment analysis of genes differentially regulated by light

Dataset S2 (XLSX)

Dataset S3. Genes differentially regulated in lnk1-1;lnk2-1 mutants compared with WT plants

Dataset S3 (XLSX)