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Article

Arabidopsis thaliana Circadian Clock Is Regulated by the Small GTPase LIP1

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Summary

Background: At the core of the eukaryotic circadian network, clock genes/proteins form multiple transcriptional/translational negative-feedback loops and generate a basic ~24 hr oscillation, which provides daily regulation for a wide range of processes. This temporal organization enhances the fitness of the organism only if it corresponds to the natural day/night cycles. Light is the most effective signal in synchronizing the oscillator to environmental cycles.

Results: The *lip1-1* (*light insensitive period 1*) mutant isolated from the model plant *Arabidopsis thaliana* displays novel circadian phenotypes arising from specific defects in the light input pathway to the oscillator. In wild-type plants, period length shortens with increasing light fluence rates and the phase of rhythms can be shifted by light pulses administered to dark-adapted plants. In contrast, in *lip1-1*, period length is nearly insensitive to light intensity and significantly larger phase shifts (delays) can be induced during the subjective night. The mutant also displays elevated photomorphogenic responses to red and blue light, which cannot be explained by the circadian defect, suggesting distinct functions for *LIP1* in the circadian light input and photomorphogenesis. The *LIP1* gene encodes a functional, plant-specific atypical small GTPase, and therefore we postulate that it acts similarly to ZEITLUPE at postranscriptional level.

Conclusions: LIP1 represents the first small GTPase implicated in the circadian system of plants. LIP1 plays a unique negative role in controlling circadian light input and is required for precise entrainment of the plant clock.

Introduction

The circadian clock is a biological timing mechanism that provides rhythmicity to gene expression, metabolism, and physiology in many organisms. This internal clock helps the organisms to anticipate the most predictable periodic environmental change on Earth: the succession of days and nights, allowing different processes to be scheduled to the most appropriate time of the day. Precise synchronization of these internal processes to rhythmically changing environmental cues has been shown to enhance fitness of organisms [1].

The genetic circuit underlying the *Arabidopsis* circadian oscillator was initially proposed to function through the reciprocal regulation between the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *TIMING OF CAB EXPRESSION 1* (*TOC1*) genes [2–4]. The morning-expressed CCA1/LHY Myb transcription factors repress the *TOC1* gene; conversely, the evening-expressed TOC1 positively regulates the transcription of *CCA1/LHY* [5]. TOC1 belongs to the PSEUDO RESPONSE REGULATOR (PRR) protein family, which consists of five members: TOC1/PRR1, 3, 5, 7, and 9 [6].

Recent results of mathematical modeling and experimental approaches have revealed two additional regulatory loops coupled to the CCA1/LHY-TOC1 circuit. The "evening loop" is formed by TOC1 and a hypothetical factor Y, both expressed in the evening. Y positively regulates *TOC1*, whereas TOC1 represses *Y* transcription, which is also inhibited by CCA1/LHY. TOC1 promotes *CCA1/LHY* transcription via another hypothetical component, X [7]. It has been demonstrated that GIGANTEA (GI), a nuclear protein with unknown biochemical function, is an essential contributor to Y function [8]. The "morning loop" is formed by CCA/LHY and PRR7/9. CCA1/LHY activates *PRR7/9* expression in the morning; conversely, PRR7/9 inhibit *CCA1/LHY* during the rest of the day [8, 9]. The coordinated function of the three loops is required to generate the ~24 hr basic oscillations in *Arabidopsis*.

This oscillation is synchronized to the environment via periodic light and temperature signals normally associated with the natural day/night cycles. Light signals are perceived by the red/far-red light-absorbing

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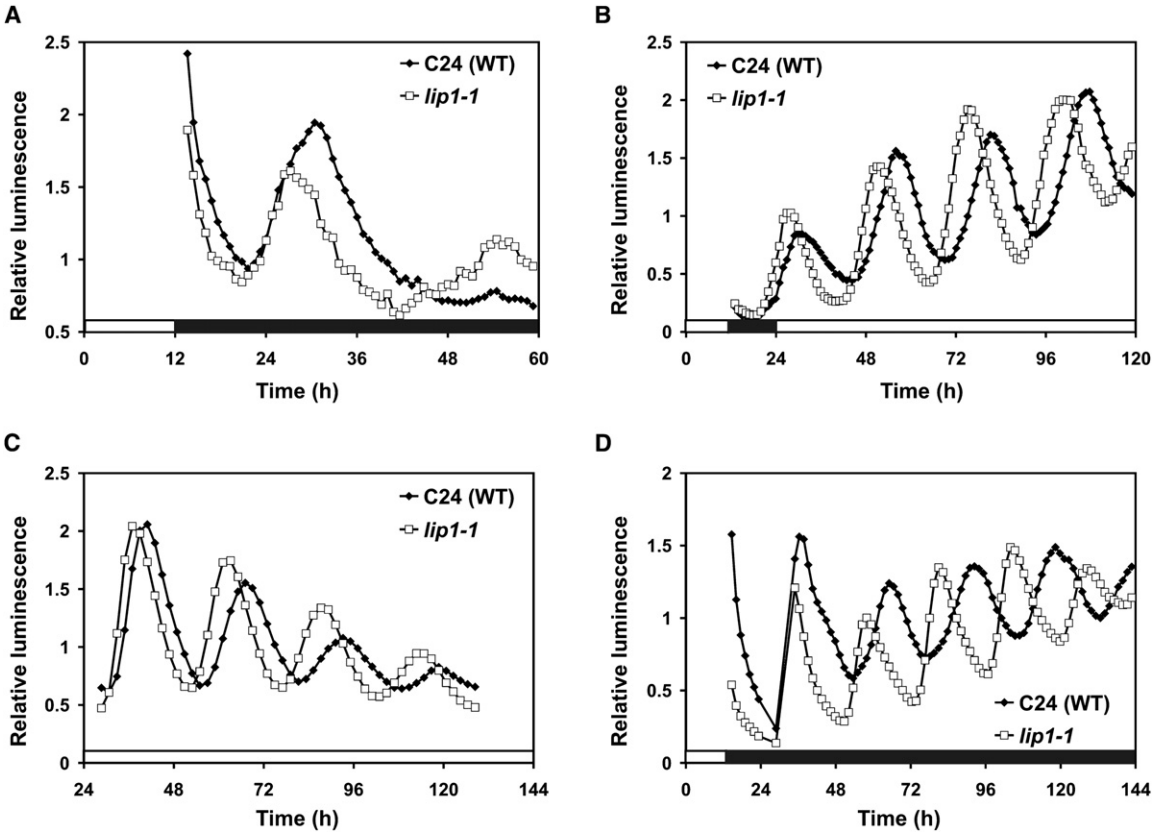

Figure 1. The *lip1-1* Mutation Shortens the Period of Circadian Expression of *CAB2:LUC* and *CCR2:LUC*+ Reporter Genes in Constant Dark and Light

Seedlings were grown in 12 hr white light (~50 μmol m^{-2} s^{-1})/12 hr dark cycles (LD 12:12) for 7 days before being transferred to constant dark at T = 12 or to constant light at T = 24. Black and white boxes represent dark and light conditions, respectively.
(A) *CAB2:LUC* rhythm in wild-type (C24) and in *lip1-1* mutant in constant darkness.
(B) *CAB2:LUC* rhythm in wild-type and in *lip1-1* mutant in constant red light (~5 μmol m^{-2} s^{-1}).
(C) *CCR2:LUC*+ rhythm in wild-type and in *lip1-1* mutant in constant red light (~5 μmol m^{-2} s^{-1}).
(D) *CCR2:LUC*+ rhythm in wild-type and in *lip1-1* mutant in constant dark.

phytochrome photoreceptors and the blue light-absorbing cryptochromes [10, 11] and are transduced to the oscillator through the input pathways. The resetting process, also called entrainment, is essential for setting the phase of the oscillator to the environmental light/dark cycles. The light input also modulates the pace/period length of the clock under constant light conditions. Each loop of the plant oscillator contains at least one light-inducible/sensitive component (*PRR9*, *CCA1/LHY*, *GI*) providing a possible molecular mechanism for resetting. The F-box protein ZEITLUPE (ZTL) [12] and protein kinase CK2 [13] represent a different level of regulation (they are not directly involved in the transcriptional control of clock genes), but they primarily affect the abundance or activity of certain clock proteins. ZTL directs TOC1 for degradation in a light-dependent manner [14], whereas CK2 modulates the activity of CCA1 via phosphorylation [15].

In this paper, we report the identification and characterization of a novel clock-associated factor, *LIGHT INSENSITIVE PERIOD 1 (LIP1)*. We demonstrate that LIP1 is a negative factor controlling the light-dependent period shortening of circadian rhythms and light-induced phase resetting during the subjective night in plants and that LIP1 represents the first small GTPase affecting the circadian clock function in plants. Small monomeric GTPases form a large family of eukaryotic proteins with a highly conserved basic biochemical function, which relies on binding and subsequent hydrolysis of guanine nucleotides in a cyclic manner [16]. Small GTPases are molecular switches shuttling between the GDP-bound inactive and the GTP-bound active states. Based on their structural and functional similarities, small GTPases are divided into five subfamilies: Ras, Rho, Rab, Ran, and Arf, respectively [17]. LIP1 owns some characteristics of the above-mentioned classes, but it exhibits remarkable differences that makes this molecule a member of a new, seed plant (Spermatophyta)-specific subfamily of small GTPases.

Results

lip1-1 Affects Multiple Circadian Clock Outputs and Period of the Clock

lip1-1 was initially isolated as an early-phase phase mutant based on the expression pattern of the morning-expressed *CAB2:LUC* circadian output marker during the first 36 hr in constant dark (DD) (Figure 1A). However,

Table 1. Period Estimation of Leaf Movement Rhythm in Continuous White Light

	Experiment 1		Experiment 2		Experiment 3	
	Period	SEM (n)	Period	SEM (n)	Period	SEM (n)
C24 (WT)	26.40	±0.28 (13)	26.95	±0.14 (22)	25.46	±0.59 (14)
lip1-1	25.81	±0.25 (9)	24.35	±0.24 (23)	24.05	±0.71 (8)

Period length data were obtained by monitoring the leaf movement rhythms in 10-day-old *lip1-1* mutant and wild-type seedlings under low intensity of continuous white light (1.5 μmol m^{-2} s^{-1}).

prolonged free-running experiments in constant red light (LL) demonstrated that the period length of *lip1-1* is 1.5–2 hr shorter than that of wild-type (WT) plants (Figure 1B). A different molecular marker, the evening-expressed *CCR2:LUC*+, also displayed a similar short-period phenotype in LL and DD (Figures 1C and 1D, respectively), and leaf movement rhythms were also shortened in *lip1-1* (Table 1). These results indicate that *LIP1* is not a component of the output pathway, because the mutation affects several different overt rhythms. To test the light dependecy of the phenotype, the rhythmic expression of *CCR2:LUC*+ was measured in etiolated WT and *lip1-1* plants entrained by 12 hr 24°C/12 hr 18°C temperature cycles for 3 days and released to constant 22°C. Both WT and *lip1-1* seedlings showed clear oscillations, but the period length of *CCR2:LUC*+ was significantly shorter in *lip1-1* (25.4 ± 0.1 hr [SEM]) than in WT (27.92 ± 0.46 hr). This result demonstrates that *LIP1* is required for normal clock function in plants grown in the complete absence of light.

In plants, there is an inverse relationship between the free-running period length in LL and the fluence rate of the light: the higher the fluence rate, the shorter the period. As a result, fluence rate curves (FRCs, plots of period values as the function of fluence rates) in plants show a negative slope [18]. To construct FRCs, WT and *lip1-1* seedlings expressing *CCR2:LUC*+ were entrained by 12:12 LD cycles for 1 week and transferred to different fluences of constant red or blue light. Figure 2 shows that period lengths in the mutant seedlings remain almost constant over the tested range of fluence rates, in contrast to WT plants, where periods are shortened by increasing light intensity. As a result, periods of WT and mutant plants were indistinguishable at medium and high fluences of red light (Figure 2A) and high fluences of blue light (Figure 2B). Moreover, period lengths in *lip1-1* plants in DD fall in the range of period values plotted on FRCs, further corroborating the fact that light has very little effect on the pace of the clock in *lip1-1* (Table 2).

lip1-1 Is More Sensitive to Resetting Light Stimuli around Dusk

Light input is important not only for tuning the period length but also for daily entrainment. It is a well-known phenomenon that the clock restricts its own sensitivity to resetting stimuli to particular times of the day, especially around dawn and dusk by rhythmic regulation of the input components. To test whether the mutation affects this function, we scanned the entrainability of the clock during a circadian cycle. Seedlings free-running in DD were irradiated for 1 hr by red light pulses (18 μmol m^{-2} s^{-1}) in every 3 hr. The phase of the circadian rhythm shifts upon these resetting light pulses. After correction by the free running period of the untreated plants, the phase shifts were plotted as a function of the time of the given resetting stimuli, yielding the phase response curves (PRCs) of *lip1-1* and WT plants (Figure 2C). The most significant difference between *lip1-1* and WT plants in sensitivity for resetting stimuli can be observed during the first half of the subjective night. This suggests an important function for LIP1 at this time of the cycle.

Analysis of Period Length and mRNA Abundance of Core Clock Components in *lip1* Mutants

To see how period length and mRNA level of some of the core oscillator components is affected by LIP1, we analyzed the temporal expression of the morning-expressed core oscillator genes *CCA1*, *LHY*, and the evening-expressed *TOC1*. The period length of the *CCA1:LUC*+ and *LHY:LUC*+ reporters was tested in WT and *lip1-1* seedlings under low fluences of red light (Figures S1A and S1B in the Supplemental Data available online). We found that the 1.5–2 hr short-period phenotype is apparent in all cases in the mutant plants by the fourth day of the free run (Table 2). The rhythmic accumulation of *CCA1*, *LHY*, and *TOC1* mRNA was measured in *lip1-2* (*lip1-2* is a T-DNA insertion null allele of *lip1* in the Col-0 accession, see later) under the same conditions by northern blot (Figures 3A–3C). This figure shows that although the mutation alters the period length of rhythmic expression, the mean levels of either the morning- or the evening-expressed core oscillator genes are not affected significantly under free-running conditions. We also defined mRNA abundance of the same clock genes by quantitative PCR in seedlings grown in LD cycles. Figures 3D and 3E show that expression patterns of *CCA1* and *LHY*, respectively, were comparable in *lip1-1* and WT seedlings. In contrast, Figure 3F illustrates that the abundance of *TOC1* mRNA was drastically reduced in *lip1-1* as compared to WT.

LIP1 Encodes an Atypical Small GTPase

Genetic mapping of the *lip1-1* mutant identified At5g64813 as *LIP1* and showed that the mutation is caused by a deletion eliminating the first half of the gene (for detailed description, see Supplemental Experimental Procedures and Figure S2) Successful complementation of *lip1-1* by expressing the YFP-LIP1 fusion protein under the control of the *35S* or the native *LIP1* promoter verified the mapping data (Figure S3). Based on the predicted amino acid sequence, LIP1 shows significant similarity to small GTPases [16, 17], but with some remarkable differences. LIP1 contains peptide inserts at several positions that are missing from the classical small GTPases. The most interesting feature is the replacement of glutamine$_{94}$ (Q_{94}) for histidine (H). This

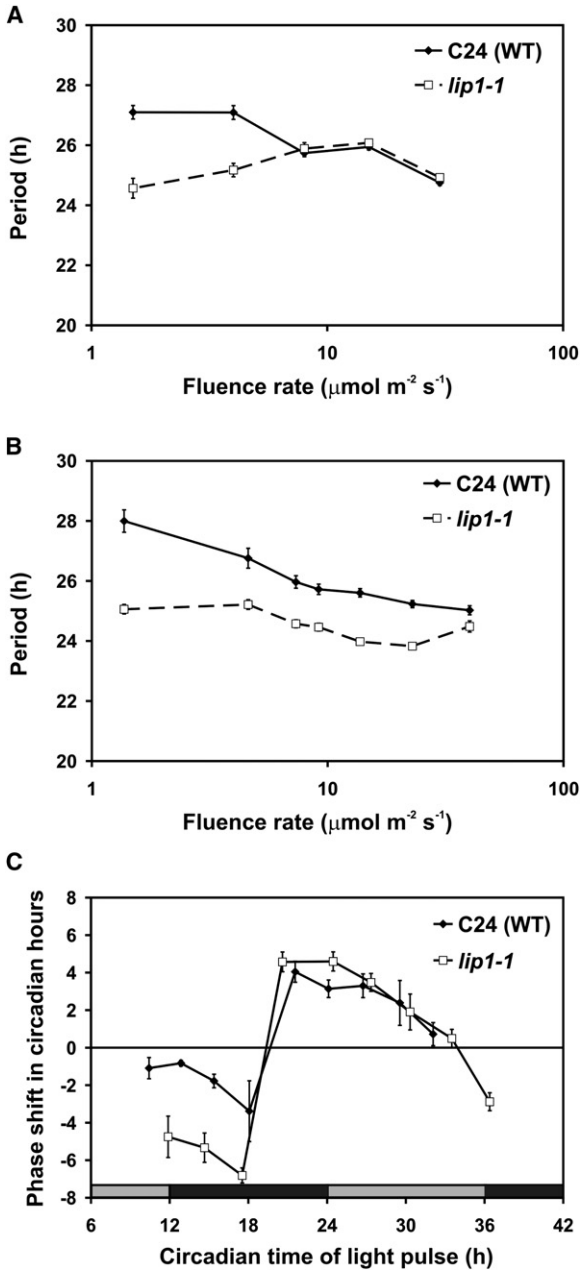

Figure 2. The *lip1-1* Mutation Affects Different Properties of the Light Input Pathway

(A and B) Fluence rate response curves were created by determining the period of *CCR2:LUC*+ expression in wild-type (C24) and *lip1-1* mutant plants under constant red (A) or blue (B) light conditions. Seedlings were entrained in LD 12:12 for 7 days prior to free run in constant light. Error bars represent standard error values.

(C) Phase response curves for wild-type (C24) and *lip1-1* plants were constructed by plotting the phase shifts of *CCR2:LUC*+ rhythm triggered by red light pulses (1 hr, 18 μmol m^{-2} s^{-1} intensity) against the circadian time of the light pulse. Phase advances and delays are shown as positive and negative values, respectively. Prior to the experiment, seedlings were grown in LD 12:12 for 7 days and then transferred to constant darkness at dusk. Grey and black boxes indicate subjective days and nights, respectively. Error bars represent standard error values.

catalytical glutamine is highly conserved in all of the classical small GTPases [19]. Its mutation results in the loss of GTPase activity (while the protein still binds GTP), leading to a constitutively active state of the protein. Finally, LIP1 lacks specific motifs at the N or C terminus for lipid modifications (Figure S4).

In order to test whether LIP1 is a functional GTPase, we purified recombinant maltose-binding protein-LIP1 (MBP-LIP1) fusion proteins from *Escherichia coli* and tested for GTP-hydrolyzing activity with [α-^{32}P]GTP as substrate. Reaction products were analyzed by thin layer chromatography. We were able to detect significant GTP hydrolyzing activity of LIP1, which clearly showes that, despite its atypical nature, LIP1 is a functional GTPase (Figure 4D).

We characterized expression pattern of *LIP1* at multiple levels. First, we showed that abundance of *LIP1* mRNA in WT seedlings in LL is not significantly affected by the circadian clock and shows only a very weak oscillatory pattern, if any (Figure S5). Second, we determined abundance of the YFP-LIP1 fusion protein in *lip1-1* seedlings complemented by expressing the *35S:YFP-LIP1* transgene under the same conditions. Results obtained by western blot analysis do not exclude a possible, low-amplitude circadian oscillation in the YFP-LIP1 protein level with a peak around the subjective dusk at ZT 15 (Figure 4A). This expression pattern conditionally supports the PRC data indicating that LIP1 may function at the first half of the subjective night.

Finally, we characterized the subcellular localization of the LIP1 protein in darkness, where it most clearly exerts its function. 4-day-old etiolated *lip1-1* mutant plants expressing the YFP-LIP1 fusion protein were analyzed by confocal microscopy. YFP fluorescence can be observed both in the nucleus and the cytosol of hypocotyl epidermal cells (Figures 4B and 4C). The nucleus was evenly filled with YFP signal; nuclear speckles were not detected. The subcellular localization of LIP1 is not significantly affected by light; essentially the same distribution pattern was observed in light-grown plants (data not shown).

Discussion

LIP1 Regulates Light Input to the Plant Circadian Clock

The function of the light input pathway is required to entrain or reset the subjective time of circadian oscillators to the local time. Based on the duration of irradiation, light affects seemingly different parameters of the oscillator. Under constant conditions, light controls the free-running period length. In diurnal organisms, like plants, the longest period is measured in DD and gradually shorter periods are observed with increasing fluence rates of light in LL (parametric entrainment, tested by FRCs) [18]. On the other hand, discrete light pulses elicit characteristic phase shifts of the oscillator free-running in darkness. In all organisms studied so far, light pulses applied during the early or late subjective night induce phase delays or advances, respectively (nonparametric entrainment, tested by PRCs) [20]. Parametric entrainment is usually explained as the net effect of phase advances and delays over the circadian cycle under constant conditions, suggesting that the underlying

Table 2. Period Estimates for Rhythmic Luciferase Reporters in Wild-Type and *lip1-1* Plants

Genotype	Reporter	Light Condition	Period (hr)	SEM	Number of Seedlings
C24 (WT)	*CCR2:LUC*	DD	27.92	±0.24	56
lip1-1	*CCR2:LUC*	DD	25.11	±0.28	55
C24 (WT)	*CCR2:LUC*	LL	27.09	±0.31	27
lip1-1	*CCR2:LUC*	LL	25.17	±0.22	24
C24 (WT)	*CAB2:LUC*	LL	28.35	±0.88	28
lip1-1	*CAB2:LUC*	LL	25.37	±0.26	56
C24 (WT)	*CCA1:LUC*	LL	27.16	±0.27	23
lip1-1	*CCA1:LUC*	LL	24.88	±0.37	22
C24 (WT)	*LHY:LUC*	LL	26.52	±0.20	23
lip1-1	*LHY:LUC*	LL	24.70	±0.57	22

Seedlings were grown under 12 hr white light/12 hr dark cycles for 7 days, then moved to constant darkness (DD) at dusk or to constant red light (LL, ~ 5 μmol m^{-2} s^{-1}) at dawn. Rhythm analysis was performed by BRASS.

mechanism of parametric and nonparametric entrainment is the same [21].

We propose that LIP1 plays a negative role in controlling circadian period and that light suppresses this effect in a fluence rate-dependent manner. Elimination of LIP1 function (e.g., *lip1-1*) mimics the effect of light and results in a short-period phenotype even in darkness. It follows that in WT plants, LIP1 function is fully suppressed at those fluence rates, where *lip1-1* plants display WT periods. We emphasize, however, that although the period length is less sensitive to light in *lip1-1*, PRCs revealed hypersensitivity to resetting light pulses in the mutant specifically during the first half of the subjective night, producing significantly larger phase delays than in WT (Figure 2). The molecular mechanism by which LIP1 negatively regulates resetting, however, remains to be elucidated.

ELF3 and ZTL are the only known clock-associated factors whose function could be paralleled to that of LIP1 in some aspects. ELF3 attenuates resetting light signals similarly to LIP1, but at a slightly later phase. ELF3 negatively regulates period length similarly to LIP1, but this is light dependent: WT periods were observed in DD in plants misexpressing *ELF3*. Moreover, ELF3 probably affects the clock via the transcription of *CCA1/LHY* [20].

ZTL has a function in regulating period length opposite to that of LIP1. *ztl* mutants show extreme long periods in DD that are dramatically shortened by light. However, ZTL affects the clock at the posttranscriptional level [14]. Based on our data, we propose that LIP1 controls the pace of the clock acting primarily at posttranscriptional level and could be involved in the regulation of the abundance or nucleo-cytoplasmic distribution of its yet unknown target.

Independent of this hypothesis, we note that the reduced level of *TOC1* mRNA in LD-grown seedlings as shown in Figure 3F is consistent with a shortening of the period in the *lip1* mutant: strong loss-of function *toc1* mutants have even shorter periods than *lip1*. Current models of the plant clock mechanism [8, 22] indicate that *TOC1* is repressed by the morning functions of both LHY and CCA1 and activated by the evening functions including GI. It remains to be determined which regulatory mechanism links LIP1 function to the clock circuit.

Light inhibition of hypocotyl elongation is rhythmically gated by the circadian clock, and virtually all clock mutants show aberrations in this photomorphogenic response [23]. Our data indicate a negative role for LIP1, throughout the entire fluence rate range tested, in red and blue (Figures 5A and 5B) but not in far-red light-dependent inhibition of hypocotyl growth (Figure 5C). Hypocotyl length in dark-grown plants was the same in all genotypes, indicating that the differences observed were indeed light dependent (Figure 5D). The hypersensitivity to red light does not depend on a functional PhyB (Figure S6) and similarly to blue light is also apparent at high fluence rates, where the clock function is not affected by *lip1-1.* Thus, our data suggest a separate role for LIP1 in photomorphogenesis. There is only one small GTPase that has been implicated in the regulation of hypocotyl elongation so far: PRA2 from pea is a typical Rab-like small GTPase [24] and was shown to modulate the synthesis of brassinosteroids in the dark. Misexpression of PRA2 in transgenic tobacco results in a dark-specific hypocotyl phenotype, indicating substantially different functions for PRA2 and LIP1 in the regulation of hypocotyl elongation.

LIP1 Represents a Novel Biochemical Function in the Circadian Clock

LIP1 belongs to a novel subfamily of small GTPases (Figure S4). In vitro assays demonstrated significant GTP binding and hydrolyzing activity of LIP1, making this protein the first small GTPase with a role in the circadian network of plants. In fact, there are at least two other small GTPase that have been implicated in the function of circadian clocks in any organisms studied so far. Mutations in *rab3a* have been suggested to affect the period length of behavioral rhythms in mice [25]. However, the core molecular oscillator was not affected, indicating that RAB3A is not functioning in the light input pathway or the oscillator itself, but probably affects the coordination/coupling of rhythm-generating nerve cells. On the other hand, DEXRAS1 has been implicated in shaping the phase-dependent responsiveness of the mammalian circadian clock to photic entrainment cues [26].

Analysis of *Arabidopsis* protein sequences identified only one close homolog (named LIP2) of LIP1 sharing the characteristic motifs. Tblastn search of available EST and genomic databases revealed the presence of highly conserved LIP-like sequences in several higher plant taxa, but not in nonplant organisms, suggesting a function for LIP-like molecules that is associated

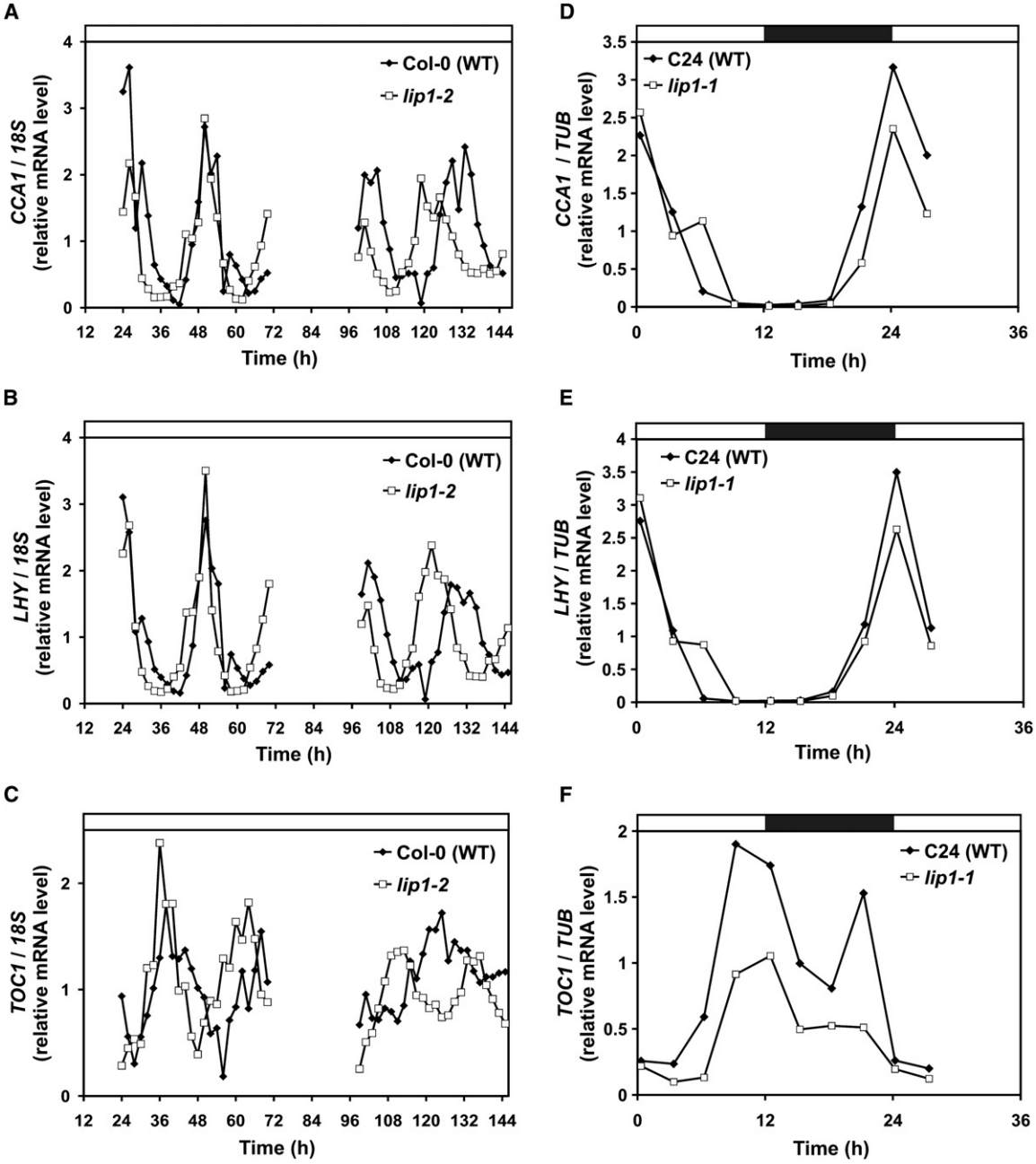

Figure 3. Mutations in *LIP1* Alter the Expression Pattern of Oscillator Components

Seedlings were grown in LD (white, 50 μmol m^{-2} s^{-1}) 12:12 for 7 days and then transferred to constant red light (5 μmol m^{-2} s^{-1}) at T = 0 (A–C). Alternatively, seedlings were grown in dim LD (white, 4 μmol m^{-2} s^{-1}) for 9 days and harvested under these conditions (D–F). Black and white boxes at the top of the charts represent dark and light conditions, respectively. Transcript levels of clock genes were determined by northern analysis (A–C) or by quantitative PCR (D–F). Gene-specific signals were normalized to the *18S* rRNA (A–C) or the *TUBULIN* (D–F) controls.

(A and D) Relative levels of *CCA1* mRNA.

(B and E) Relative levels of *LHY* mRNA.

(C and F) Relative levels of *TOC1* mRNA.

The measurements were repeated 2 to 3 times and one representative set of data is shown on each panel.

with the physiology of seed plants (Spermatophyte; Figure S4).

Conclusions

We identified the small GTPase LIP1 as a novel component of the light input pathway of the plant circadian network. We demonstrate that LIP1 function is required for the light-dependent modulation of period length and for proper resetting of the clock by light pulses, especially during the early subjective night. Our data show that LIP1 limits the degree of phase resetting by light pulses at this time of the circadian cycle, when light is normally not present. It is possible that LIP1 protects the clock from excessive or mistimed light and,

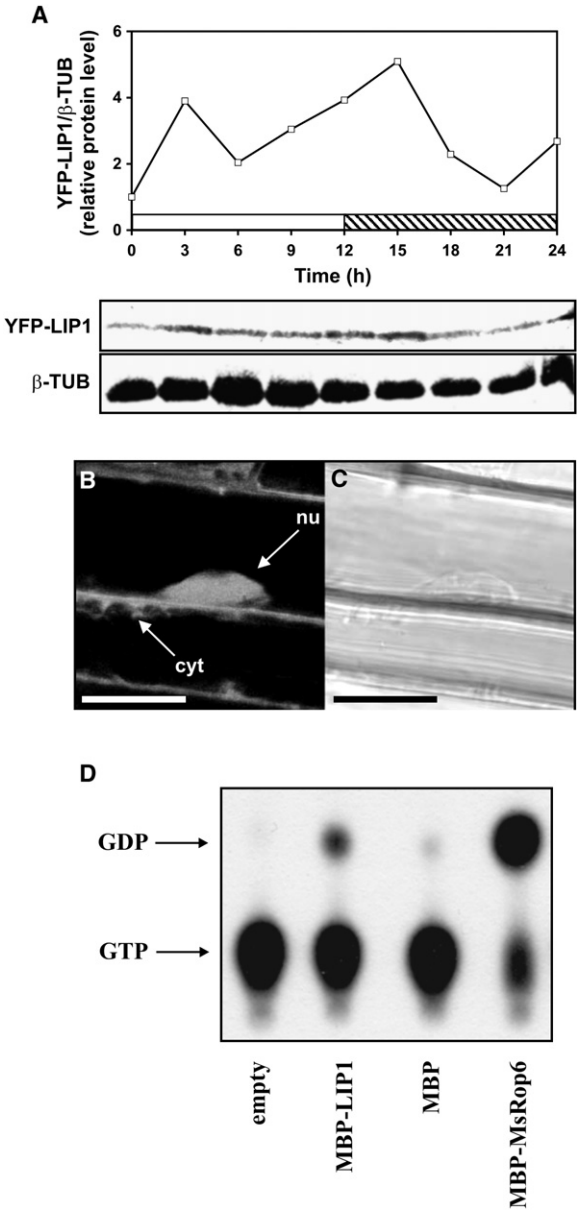

Figure 4. Functional Characterization of the LIP1 Protein

(A) *lip1-1* plants expressing the YFP-LIP1 fusion protein were grown in LD 12:12 for 7 days and then transferred to constant light (white, 50 μmol m^{-2} s^{-1}) at dawn (T = 0). The striped box along the x axis represents subjective night. Samples were harvested every 3 hr, and the abundance of the YFP-LIP1 fusion protein was determined by western blotting. YFP-specific signals were normalized to β-tubulin-specific signals and then to values at T = 0. Image of the corresponding blot is shown below the chart.

(B and C) Confocal image (B) and differential interference contrast image (C) of an epidermal cell in the hypocotyl near to the hook of a 4-day-old etiolated *Arabidopsis* seedling expressing the YFP-LIP1 fusion protein in the *lip1-1* mutant background. The arrows show fluorescence in the nucleus (nu) and in the cytosol (cyt). Scale bars represent 10 μm.

(D) Recombinant proteins were fused to the maltose binding protein (MBP) and expressed in bacteria. After purification, they were assayed for GTPase activity by [α-^{32}P]GTP. Reaction products were separated on silica TLC plates and visualized in a PhosphorImager. *Medicago sativa* Rop6 and MBP proteins were used as positive and negative controls, respectively. A sample with no added protein is also shown (empty). The appearance of GDP in the reaction indicates GTPase activity for the MBP-LIP1 and the positive control.

therefore, contributes to the robustness and accuracy of the plant circadian clockwork.

Experimental Procedures

Plant Materials and Growth Conditions

The *lip1-1* mutant allele was isolated from EMS-mutagenized populations of the C24 accession carrying the *CAB2:LUC* reporter gene [27]. Seeds of the T-DNA insertional *lip1-2* allele (labeled as SAIL_1157_A08) established in Columbia ecotype were obtained from the Syngenta *Arabidopsis* Insertion Library (SAIL) through The Nottingham *Arabidopsis* Stock Centre (NASC), the *LIP1* cDNA clone was obtained from RIKEN (Tsukuba, Japan) [28]. The mutant lines were backcrossed three times to the corresponding parental lines; the mutations segregated as single, recessive loci. Selection of homozygous *lip1-1* mutants from segregating lines was carried out by allele-specific PCR with the following primer set: 5′-cccgaaatgcgtcagactaagattg-3′, 5′-ttccactgcttccttttgttccttct-3′, 5′-ccttcgaatttacttactagtgtttgagat-3′. The *CCR2:LUC*+, *CCA1:LUC*+, *LHY:LUC*+ reporter gene constructs were transformed into the mutant and wild-type lines via *Agrobacterium*-mediated transformation [29]. Surface-sterilized seeds were grown in 12 hr white light (~50 μmol m^{-2} s^{-1})/12 hr dark cycles at 22°C for 7 days before being transferred to continuous darkness at ZT 12 or to continuous light at ZT 0, and all measurements were carried out at constant 22°C. ZT0 is defined as the time of the last dark-light transition before transfer to constant conditions. Illumination was provided by cool-white fluorescent tubes or monochromatic LED light sources (red, λ_{max} = 667 nm; blue, λ_{max} = 470 nm; far-red, λ_{max} = 730 nm).

Analysis of Luminescence and Leaf Movement Rhythms

Luciferase activity was measured by low-light video imaging of groups of 5–10 seedlings or by measuring single seedlings with an automated luminometer for 2–7 days as described previously [30]. For FRCs, circadian periods of *CCR2:LUC*+ activity were measured in seedlings transferred to constant illumination of red or blue light at the fluence rates indicated. For determining the circadian period of etiolated, temperature-entrained plants, seedlings were grown in darkness under 12 hr 22°C/12 hr 26°C for three cycles and transferred to constant 22°C at the time of the predicted warm-cold transition for luminescence imaging. Leaf movement rhythms were measured as described [23]. All rhythm data were analyzed with the Biological Rhythms Analysis Software System (BRASS [31], available at http://www.amillar.org), running fast Fourier transform nonlinear least-squares estimation [32]. Variance-weighted mean periods within the circadian range (15–40 hr) and SEMs were estimated as described [30], from 10 to 36 traces per genotype. Phase response curves were created and calculated as described previously [33].

Analysis of Gene Expression

Total RNA extraction, northern blotting, and quantification of *CCA1*, *LHY*, *TOC1*, and *18S* rRNA specific signals were performed as described [33]. Full-length cDNA fragments were used for *CCA1*, *LHY*, and *TOC1* hybridizations. Two independent northern analyses were performed with each probe. All graphs show mRNA levels relative to the *18S* rRNA transcript in wild-type (WT) and *lip1-2* mutant plants. Protein extracts were prepared according to Bauer et al. [34]. Quantitative PCR experiments were performed essentially as described [35]. For detailed description and primer sequences, see Supplemental Experimental Procedures.

Measurement of the Hypocotyl Length, Microscopy

For hypocotyl length measurements, seeds were sown on half MS media with 1% agar and incubated in the dark for 48 hr at 4°C. Cold-treated seeds were then irradiated with 6 hr of white light and then transferred to 22°C dark for an additional 18 hr. After this treatment, seedlings were grown at different fluences of light for 4 days. Measurement of the hypocotyl length was performed by MetaMorph Software (Universal Imaging, Downingtown, PA). Hypocotyl lengths of light-grown seedlings were normalized to the corresponding dark-grown hypocotyl length. FRCs for hypocotyl elongation were obtained by plotting relative hypocotyl lengths against the light intensities used in the experiment displayed on a logarithmic

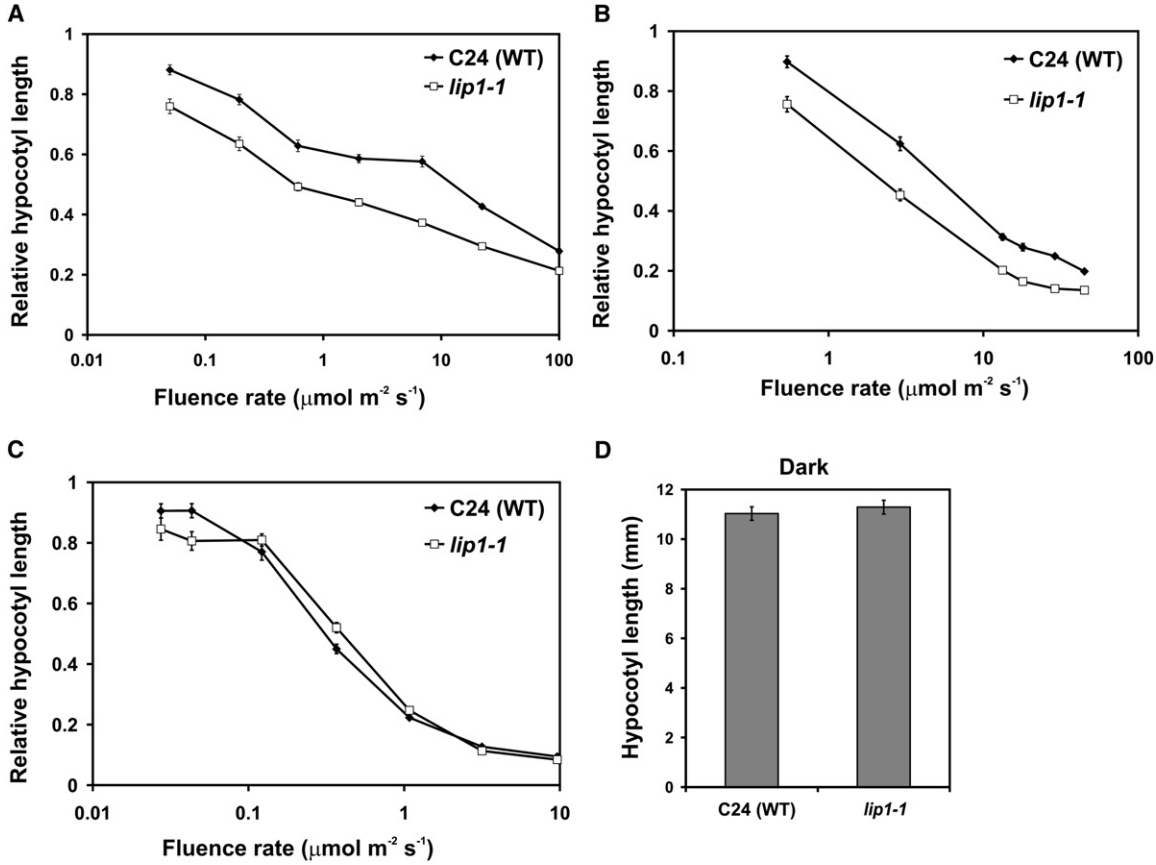

Figure 5. LIP1 Is a Negative Regulator of Photomorphogenic Responses in Young Seedlings

Seedlings were grown in constant red, far-red, or blue light at the indicated fluence rates of light for 4 days, and then hypocotyl lengths of the seedlings were measured.

(A) FRC of hypocotyl elongation in wild-type (C24) and *lip1-1* mutant seedlings in red light.

(B) FRC of hypocotyl elongation in wild-type (C24) and *lip1-1* mutant seedlings in blue light.

(C) FRC of hypocotyl elongation in wild-type (C24) and *lip1-1* mutant seedlings in far-red light.

(D) Absolute hypocotyl length of dark-grown C24 (WT) and *lip1-1* seedlings.

Error bars represent standard error values.

scale. Subcellular distribution of YFP-LIP1 protein was analyzed in 4-day-old seedlings, as described previously [34].

Recombinant Protein Purification and GTPase Activity Assay

MBP fusion proteins were expressed in BL21 Rosetta strains of *Escherichia coli* (Novagen). Intrinsic GTPase activity of purified proteins was assayed in 20 μl reaction volume containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, 10 μM cold GTP, 0.7 μCi of [α-^{32}P]GTP, and 4 μM protein. Reactions were incubated for 10 hr at room temperature. 1 μl aliquots were spotted on silica TLC plates with glass support (Merck, Silica gel 60 F_{254}). Reaction products were separated in 1-propanol:cc.NH_4OH:H_2O (11:7:2). Dried TLC plates were visualized by a PhosphorImager device.

Supplemental Data

Six figures and Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/17/17/1456/DC1/.

Acknowledgments

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Supplemental Data

Arabidopsis thaliana Circadian Clock Is Regulated by the Small GTPase LIP1

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Supplemental Experimental Procedures

Molecular Cloning Procedures

To create *LHY:LUC+* fusion construct, a 1812 bp region upstream of the *LHY* coding sequence was amplified from Columbia ecotype by PCR with ExTaq polymerase blend (TaKaRa) and the following primers: 5′-tgcggtcgactgtttcaaataactgttatgtccta-3′, 5′-ggaaggatccaacaggaccggtgcagctat-3′. PCR product was cloned directly into a pPCVH *LUC+* plant binary vector as SalI-BamHI fragment. For the complementation experiments, *LIP1:LIP1* and *$(35S)_{2X}$:YFP-LIP1* gene constructs were created. Full-length *LIP1* cDNA was obtained from RIKEN full-length cDNA library (pda02083). With this clone as a template, the *LIP1* CDS was amplified by Pfu polymerase (Promega) with the following primers: 5′-tcgcggatccgtatgaagttttggagggaacgtgaaag-3′, 5′-tcgggagctctcagacgttaatatccattcgctttgac-3′. The PCR product was cut by BamHI and SacI and cloned into pBluescript II SK(-) cloning vector (Stratagene). *LIP1* promoter was obtained by amplifying a 1137 bp region upstream of the *LIP1* coding sequence from Columbia ecotype with ExTaq polymerase blend and the following primers: 5′-cccagaattccatcctcgttgagattgtaagatacc-3′, 5′-cgccgtcgacttttgcttaacaagagcagattaggt-3′. PCR product was cloned into pBluescript as an EcoRI-*SalI* fragment. *LIP1:LIP1* was assembled in pPCVH vector (plant selection marker: hygromycin). First, *LIP1* promoter was subcloned between EcoRI and SalI sites, and then *LIP1* CDS was inserted downstream between BamHI and SacI sites. To create *LIP1* overexpressor constructs, *LIP1* CDS was subcloned into pPCVB $(35S)_{2X}$ plant binary vector (plant selection marker Basta) downstream of the $(35S)_{2X}$ promoter with BamHI and SacI sites. A *YFP* variant lacking STOP codon was inserted in frame as SalI-BamHI fragment upstream of the *LIP1* CDS to produce tagged version of the LIP1 protein for fluorescent microscopy.

To create Maltose Binding Protein (MBP)-LIP1 fusion protein, *LIP1* CDS was reamplified by Pfu polymerase with the following primers: 5′-cttttgcgaattcatgaagttttggagggaacgt-3′, 5′-tttgcgctcgagtcagtcgacgacgttaatatccattcgctt-3′. PCR product was cut by EcoRI and XhoI and ligated into EcoRI and SalI sites of the bacterial expression vector pMAL c2X (New England Biolabs). MBP-MsRop6 construct was created as follows: SalI site of the pENTR 2B was blunted by T4 polymerase and then ligated back to change the frame. After this, MsRop6 CDS was subcloned into pENTR 2Bmod between EcoRI and XhoI sites. From here, the CDS was transferred into a Gateway-compatible pMAL c2X Destiny vector by LR cloning (Invitrogen).

qPCR Primers

TOC1 RT-PCR F [S1], 5′-atc ttc gca gaa tcc ctg tga ta-3′; TOC1 RT-PCR R [S1], 5′-gca cct agc ttc aag cac ttt aca-3′; CCA1 RT-PCR F [S1], 5′-ctg tgt ctg acg agg gtc gaa-3′; CCA1 RT-PCR R [S1], 5′-ata tgt aaa act ttg cgg caa tac ct-3′; LHY RT-PCR F [S1], 5′-caa cag caa caa caa tgc aac tac-3′; LHY RT-PCR R [S1], 5′-aga gag cct gaa acg cta tac ga-3′; TUB2/3-RT-F [S2], 5′-cca gct ttg gtg att tga ac-3′; TUB2/3-RT-R [S2], 5′-caa gct ttc gga ggt cag ag-3′.

Protein Extraction and Western Blot Analysis

For protein purification, approximately 100 mg of plant material was harvested in 1.5 ml test tubes and frozen in liquid nitrogen. After homogenization with a pestle, 300 μl warm extraction buffer (4 M urea, 5% SDS, 16.66% glycerol, 5% β-mercaptoethanol) was added and ground further until no visible tissue pieces remained. Samples were incubated at 60°C for 10 min, then centrifuged at full speed for 30 min. Clear supernatant was transferred to a new tube and loaded directly on 10% SDS polyacrylamide gel. Run gels were electroblotted onto Immobilon-P PVDF membranes (Millipore). Separate membranes were probed either with monoclonal anti-GFP antibody (Clontech, Living Colors A.v. Monoclonal Antibody JL-8) in 1:1000 dilution or with polyclonal anti-β-tubulin (Sc-34002, Santa Cruz Biotechnology) antibody in 1:500 dilution. Having applied alkaline phosphatase-conjugated secondary antibodies (Sigma, anti-mouse IgG A-3562, anti-goat IgG A-4187) colorimetric assay was conducted and the dried blots were scanned with a flat bed scanner. Densitometric analysis was performed with MetaMorph Software (Universal Imaging, Downingtown, PA). The YFP signal was normalized to the β-tubulin signal measured from the same sample.

Supplemental References

S1. Edwards, K., Anderson, P., Hall, A.J.W., Salathia, N., Locke, J.C.W., Lynn, J., Straume, M., Smith, J., and Millar, A.J. (2006). FLOWERING LOCUS C mediates natural variation in the high-temperature response of the *Arabidopsis* circadian clock. Plant Cell *18*, 639–650.

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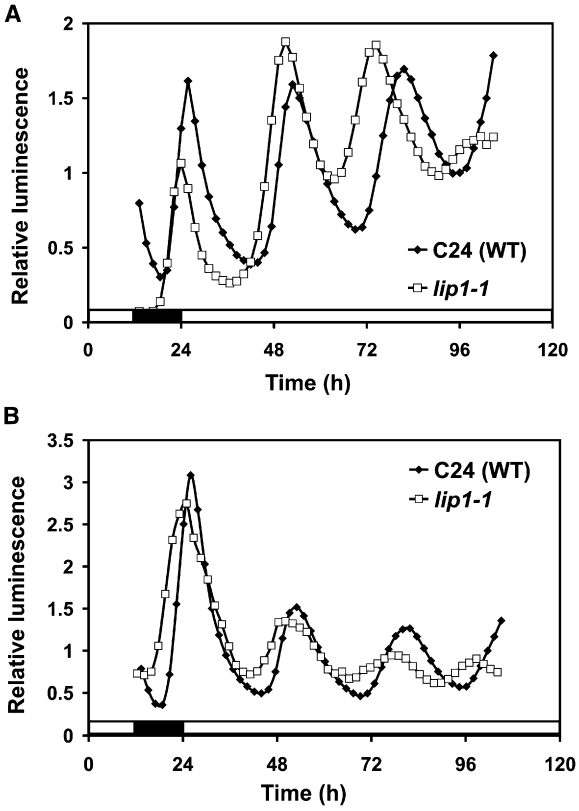

Figure S1. Mutations in *LIP1* Alter the Expression Pattern of Oscillator Components

Seedlings were grown in LD 12:12 for 7 days and then transferred to constant red light (~5 μmol m^{-2} s^{-1}) at T = 24. Black and white boxes represent dark and light conditions, respectively.

(A) Rhythmic expression of *CCA1:LUC+* reporter gene in wild-type (C24) and *lip1-1* plants.

(B) Rhythmic expression of *LHY:LUC+* reporter gene in wild-type (C24) and *lip1-1* plants.

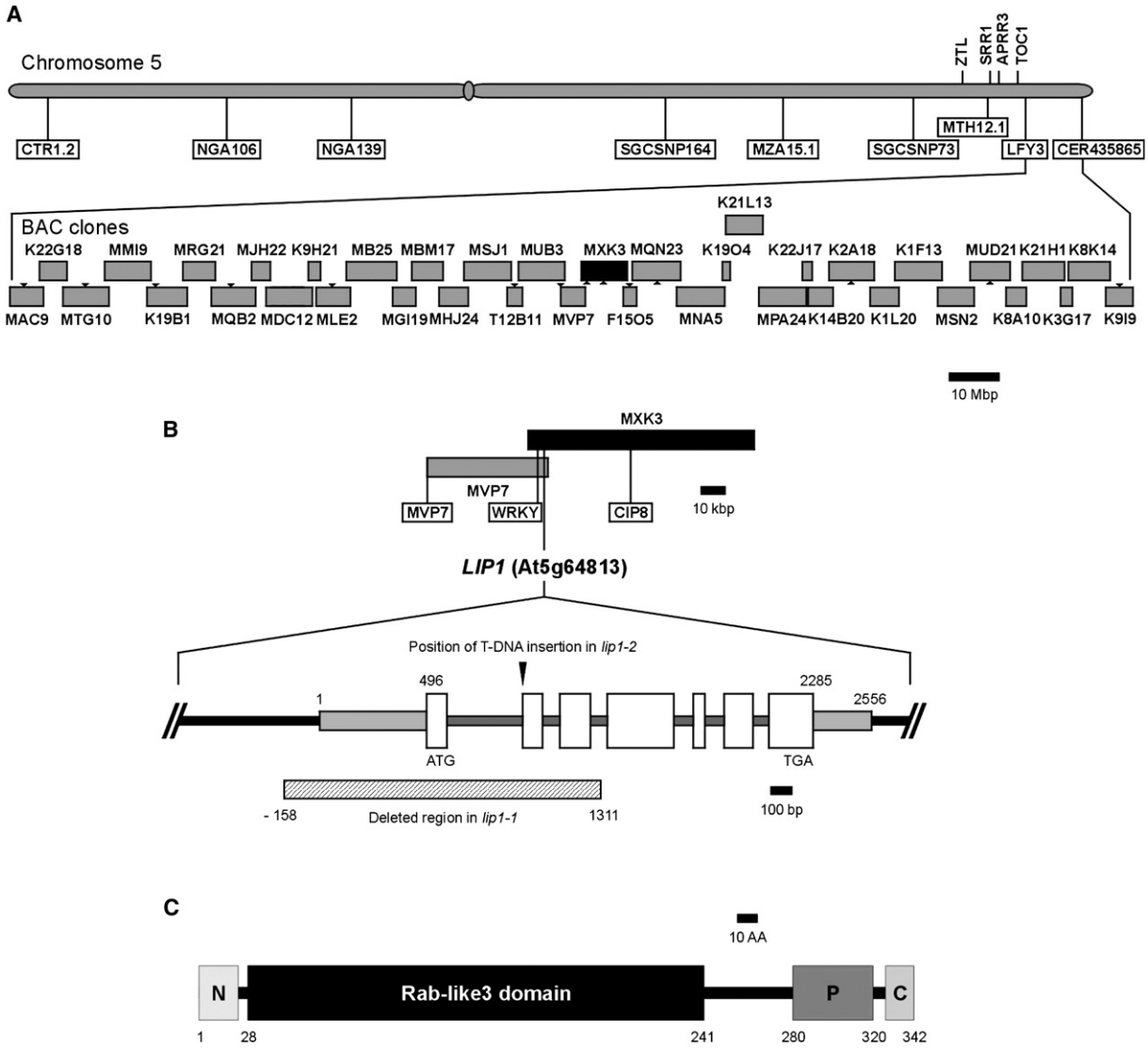

Figure S2. Map-Based Cloning of *LIP1* Gene

(A) Genetic mapping of *lip1-1* localized *LIP1* to the bottom of chromosome 5, to the region containing BACs MVP7 and MXK3. Marker names in boxes indicate the coarse mapping markers. After locating the *lip1-1* between markers SGCSNP73 and CER435865, genes with well-known circadian function (*ZTL*, *SRR1*, *APRR3*, *TOC1*) in this region were sequenced. Having found no difference in nucleotide sequences, we proceeded with fine mapping. Small triangles mark the physical location of the fine mapping markers on the BACs.

(B) With MVP7, WRKY, and CIP8 markers, the mutation was delimited between WRKY and CIP8. Sequencing of the MXK3 BAC between these two markers in *lip1-1* and C24 revealed a 1469 bp deletion in the predicted gene At5g64813 in *lip1-1*. To verify the function of this gene, an independent T-DNA insertion mutant allele (in Col-3 ecotype) was obtained from Syngenta (SAIL_1157_A08; *lip1-2*). We did not detect full-length mRNA of *LIP1* in either homozygous mutant alleles by northern blot analysis (data not shown).

(C) Schematic domain structure of the LIP1 protein. The Rab-like3 type core GTPase domain spans two-thirds of the molecule. It contains the essential residues for GTP-binding and some LIP-specific features, for example a serine-rich insert between the predicted switch I and II regions and the characteristic GHERY-motif. N and C denote distinctive N- and C-terminal sequences, respectively; P stands for a proline-rich conserved region.

A

Promoter

tccaaatctctcatcctcgttgagattgtaagataccaacaatattaatattataatctcaatgctttgtataatttgataggtgt
ataataaaggagttacttatttcgtggttataaatgtttaatgtatgtatattgctatgagtacattttgtaggtcactgactcac
tagatataaaatcttaagcgtatatttatttaaaaagtagtgttgaatttttttgggtcacggttgtccaaaatactgcgatctga
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tgacccgaaatgcgtcagactaagattgtaccgggaaggaaatgtgaaaaaagcaacttgtaaaaaaagcctgtgtgacagctctg
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tatgatccggttttggtgcgttgtgtagattgtcccgaaccgatctcatcttctacctttcaacaacttaagcaaggaaacgaagt
cgtttcatatctgttcttccgcccaaatctcaaacactagtaagtaaattc

5'-UTR

gaagggagtcttggatctctctgatctcgatccagattctctctacttctcttatcttcgatagagaaatttgtatcttcagattt
attcgaggaggaagacgaaccctacttgattgatttaggaggtggagagatctttctgaacaagtttgattctgttggtaattgat
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ggaagttgattgaaacagtgttagttttcttattttgatacctaatctgctcttgttaagcaaaa

insertion site of T-DNA in *lip1-2*

CDS

ATGAAGTTTTGGAGGGAACGTGAAAGGGAAAATAAGGAGCAGATCTTAGCGCCATTATGTGGGCAAGTTCGAGTGTTGGTAGTTGG
TGACTCAGgtactataacaagttatagtttcatgtagatcgttagatttgattacctatttttatggtatcaaaccaagttagaaat
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TGAACTCTGGGATGTATCAGGGCATGAGAGATACAAAGATTGCCGGTCACTGTTCTATTCACAAATCAATGgtctctcccaaccta
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ctttgttgggtttggtctttacggttgattgttgcagTTTCCATGGAGATCCATACAAGTACAACAACACAATACCACCACTTCCA
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TCTTTCCAGTGTACAAGAAACAACCAACAATGGCAGTGCCAGGTCAAAGCGAATGGATATTAACGTC**TGA**

3'-UTR

agtctctcatctcttgttagctcttctcattccaagaaaactttatgttgcttttggatctctattattctttttgattcttcttt
gtaggttggtaaagtgaatatgtgtgcttaacccctctttgatttgtacattgtggggtttgatcttatagcaaattctttgaatg
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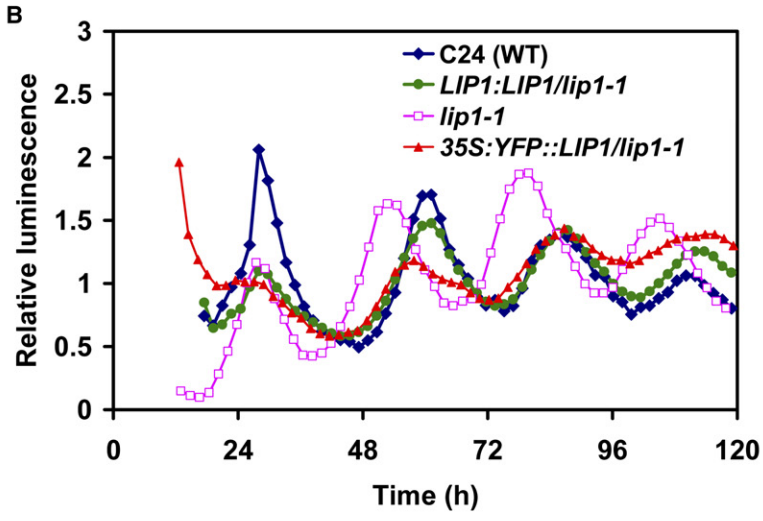
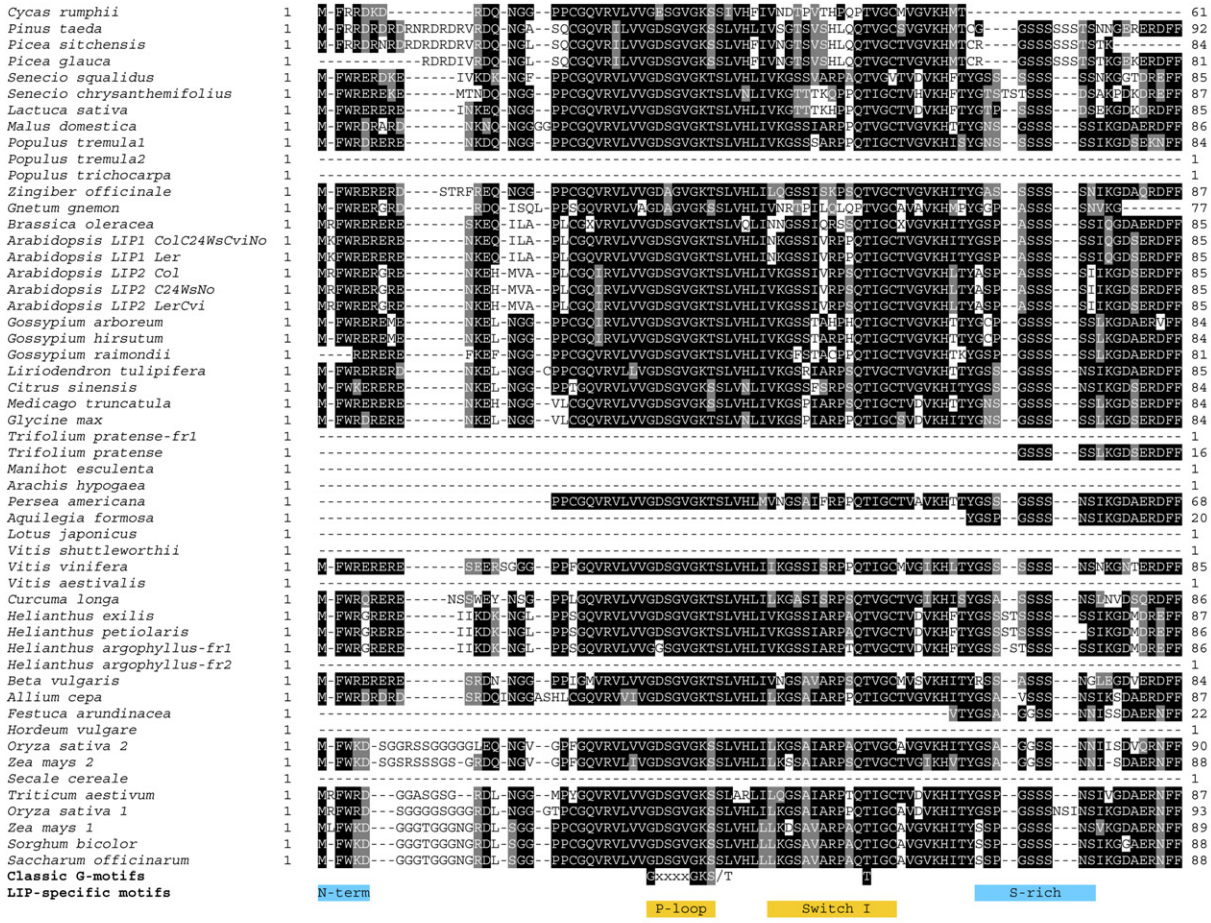


Figure S3. *LIP1* cDNA Complements the Circadian Phenotype of *lip1-1*

(A) Nucleotides that were deleted from the *LIP1* gene in the *lip1-1* mutant are shaded. Capital letters mark exons; small letters mark introns. The insertion site of the T-DNA that disrupts *LIP1* gene in the *lip1-2* mutant is shown.

(B) Circadian rhythm of *CAB2:LUC* expression in the wild-type (C24), in the *lip1-1* mutant and in LIP1 and YFP-LIP1 expressing *lip1-1* plants in constant red light (~5 μmol m^{-2} s^{-1}).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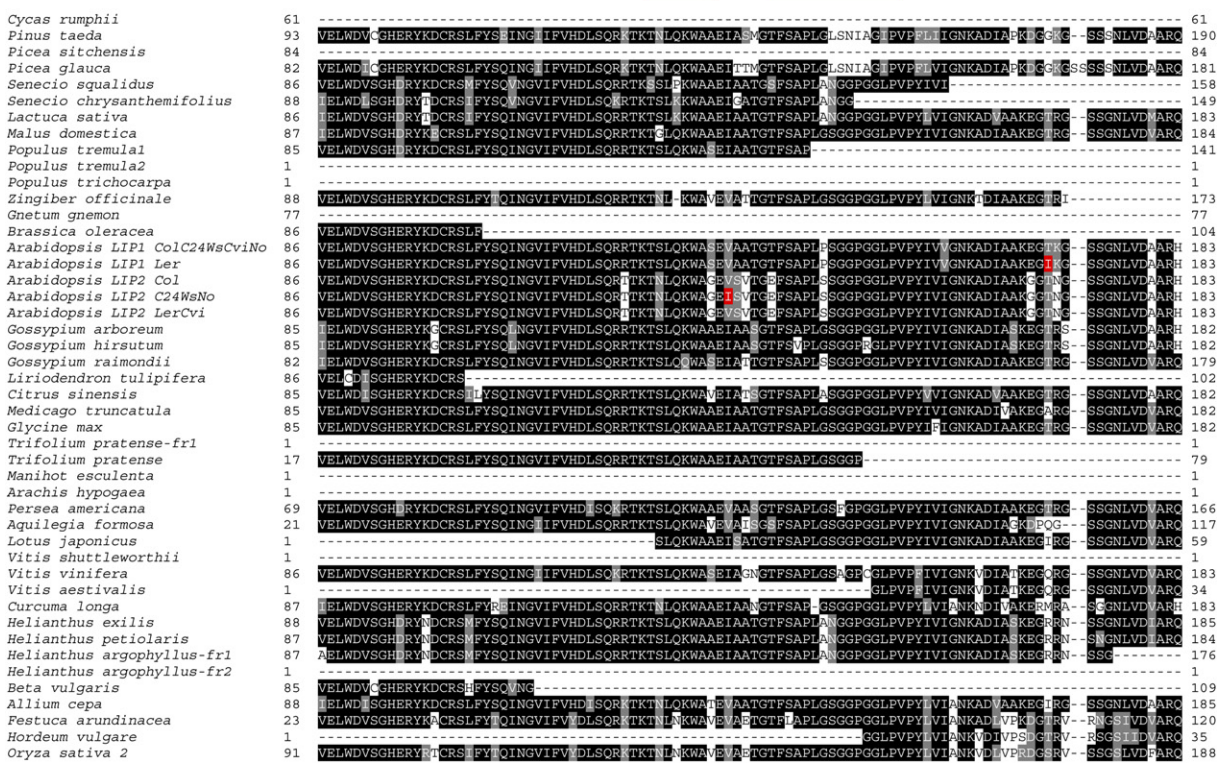
Pinus taeda
Picea sitchensis
Picea glauca
Senecio squalidus
Senecio chrysanthemifolius
Lactuca sativa
Malus domestica
Populus tremula1
Populus tremula2
Populus trichocarpa
Zingiber officinale
Gnetum gnemon
Brassica oleracea
Arabidopsis LIP1 ColC24WsCviNo
Arabidopsis LIP1 Ler
Arabidopsis LIP2 Col
Arabidopsis LIP2 C24WsNo
Arabidopsis LIP2 LerCvi
Gossypium arboreum
Gossypium hirsutum
Gossypium raimondii
Liriodendron tulipifera
Citrus sinensis
Medicago truncatula
Glycine max
Trifolium pratense-fr1
Trifolium pratense
Manihot esculenta
Arachis hypogaea
Persea americana
Aquilegia formosa
Lotus japonicus
Vitis shuttleworthii
Vitis vinifera
Vitis aestivalis
Curcuma longa
Helianthus exilis
Helianthus petiolaris
Helianthus argophyllus-fr1
Helianthus argophyllus-fr2
Beta vulgaris
Allium cepa
Festuca arundinacea
Hordeum vulgare
Oryza sativa 2
Zea mays 2
Secale cereale
Triticum aestivum
Oryza sativa 1
Zea mays 1
Sorghum bicolor
Saccharum officinarum
Classic G-motifs
LIP-specific motifs
N-term
P-loop
Switch I
S-rich


Cycas rumphii
Pinus taeda
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Picea glauca
Senecio squalidus
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Malus domestica
Populus tremula1
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Populus trichocarpa
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Gnetum gnemon
Brassica oleracea
Arabidopsis LIP1 ColC24WsCviNo
Arabidopsis LIP1 Ler
Arabidopsis LIP2 Col
Arabidopsis LIP2 C24WsNo
Arabidopsis LIP2 LerCvi
Gossypium arboreum
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Gossypium raimondii
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Citrus sinensis
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Glycine max
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Persea americana
Aquilegia formosa
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Vitis shuttleworthii
Vitis vinifera
Vitis aestivalis
Curcuma longa
Helianthus exilis
Helianthus petiolaris
Helianthus argophyllus-fr1
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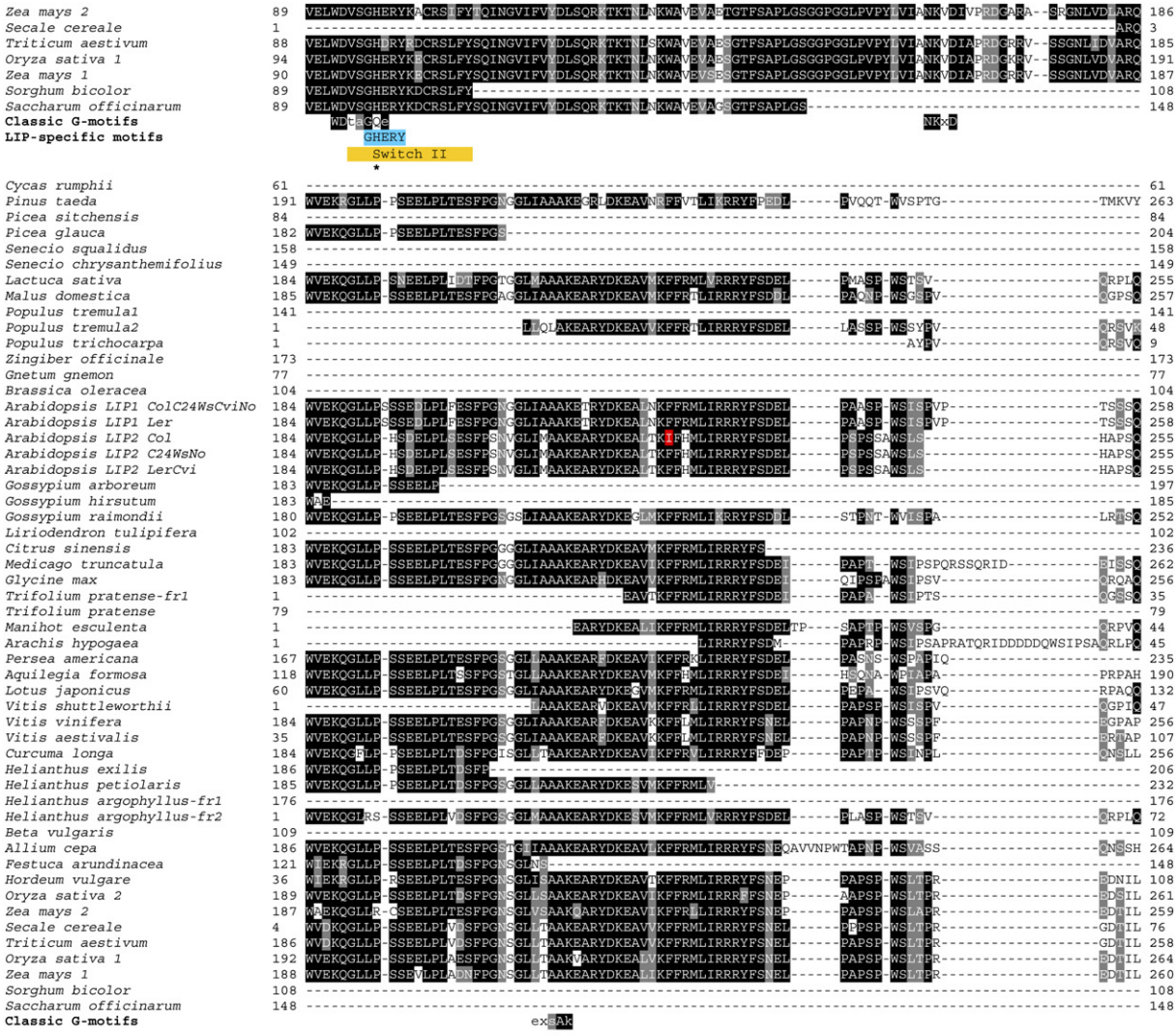
Beta vulgaris
Allium cepa
Festuca arundinacea
Hordeum vulgare
Oryza sativa 2

(Continued)


Classic G-motifs
LIP-specific motifs
GHERY
Switch II
Classic G-motifs

(Continued)

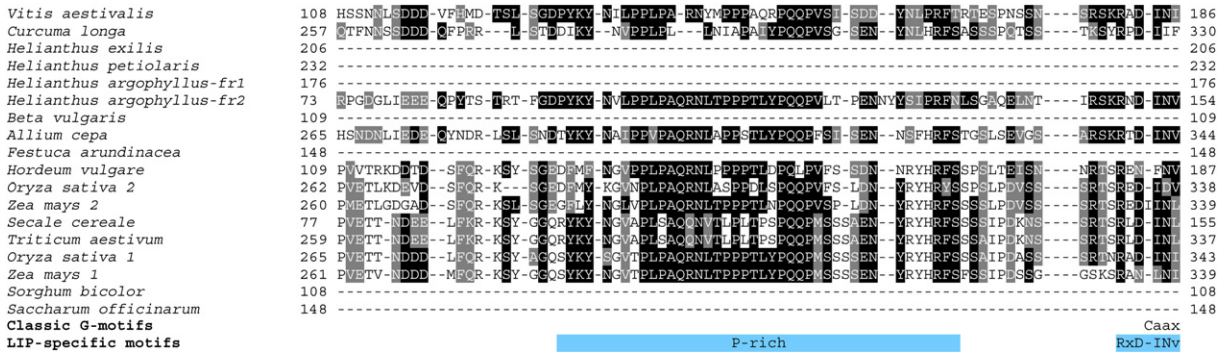


Figure S4. LIP-like Sequences from Different Plant Species

Several sequences were found in EST and genomic databases that show strong similarity to the AtLIP1 molecule. These molecules contain the Rab-like3 (RabL3) domain that lacks some conserved residues of the classical small GTP-binding proteins. These conserved motifs responsible for GTP/GDP binding, GTPase activity, and lipid modification in classical small GTP-binding proteins are indicated under the aligned sequences. The unusual H residue instead of the conserved Q is marked by an asterisk. This residue ("GHERY"-motif) defines a plant-specific subgroup within the RabL3 family of proteins. The core GTPase domain is interrupted between the predicted switch I and II regions by a serine-rich insert that may serve as a target for regulatory kinases. LIP-like molecules have a distinctive N-terminal end and a C-terminal extension with a characteristic proline-rich region. At the C-terminal end, a conserved RxDINv motif can be found instead of the classical Caax lipid modification motif. Amino acid substitutions identified in different *Arabidopsis* ecotypes are marked red. Note that in the Col-0 variant of the AtLIP2 a very conserved phenylalanine (213) was changed to isoleucine.

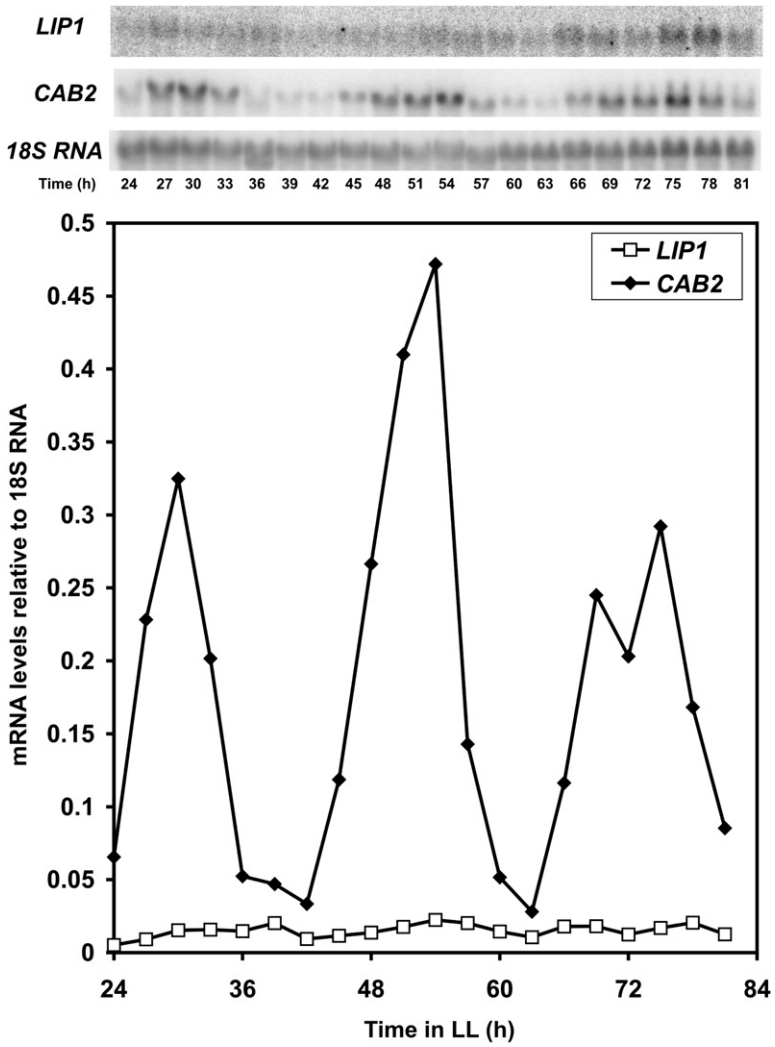


Figure S5. *LIP1* mRNA Levels Are Not Regulated by the Circadian Clock

C24 (WT) seedlings were grown in 12 hr white light (~50 μmol m^{-2} s^{-1})/12 hr dark cycles (LD 12:12) for 7 days before being transferred to constant light (white, ~50 μmol m^{-2} s^{-1}) at T = 0. Samples were harvested at times indicated. mRNA abundance of *LIP1*, *CAB2*, and *18S RNA* genes was measured by northern blotting. *LIP1* and *CAB2* specific signals were normalized to the *18S RNA*-specific signals and plotted against the time of harvesting.

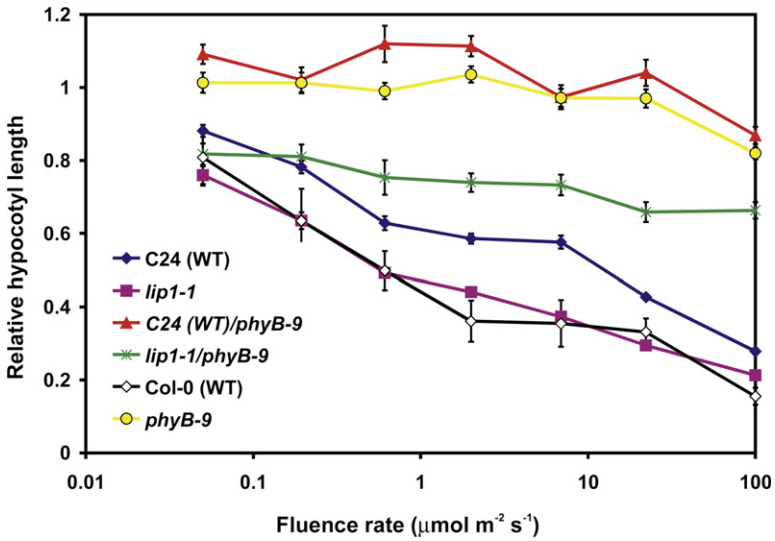


Figure S6. Photomorphogenic Phenotype of *lip1-1* in Constant Red Light Does Not Depend on Functional PhyB Photoreceptor

lip1-1 (C24 accession) was crossed with the PhyB null mutant *phyB-9* (Col-0 accession). Homozygous double mutants (*lip1-1*/*phyB-9*) and homozygous *phyB-9* segregants with WT copies of LIP1 (C24 (WT)/*phyB-9*) along with C24 and Col-0 WT controls, and the *phyB-9* single mutant were tested for hypocotyl elongation under different fluences of constant red light. Error bars represent standard error values. The fluence rate response curves were constructed as in Figure 2.