Developmental Cell, Vol. 2, 629–641, May, 2002, Copyright ©2002 by Cell Press

LHY and *CCA1* Are Partially Redundant Genes Required to Maintain Circadian Rhythms in *Arabidopsis*

Tsuyoshi Mizoguchi,[1,5] Kay Wheatley,[1,5] Yoshie Hanzawa,[1] Louisa Wright,[1,2] Mutsuko Mizoguchi,[1] Hae-Ryong Song,[3] Isabelle A. Carré,[3] and George Coupland[1,2,4]

[1]John Innes Centre
Colney Lane
Norwich NR4 7UH
United Kingdom

[2]Max Planck Institute for Plant Breeding
Carl von Linne Weg 10
D-50829 Cologne
Germany

[3]Department of Biological Sciences
University of Warwick
Coventry CV4 7AL
United Kingdom

Summary

Several genes are known to regulate circadian rhythms in *Arabidopsis*, but the identity of the central oscillator has not been established. LHY and CCA1 are related MYB-like transcription factors proposed to be closely involved. Here we demonstrate that, as shown previously for *CCA1*, inactivation of *LHY* shortens the period of circadian rhythms in gene expression and leaf movements. By constructing *lhy cca1-1* double mutants, we show that *LHY* and *CCA1* are partially redundant and essential for the maintenance of circadian rhythms in constant light. Under light/dark cycles the *lhy cca1-1* plants show dramatically earlier phases of expression of *GI* and *TOC1*, genes associated with the generation of circadian rhythms and the promotion of *LHY* and *CCA1* expression. We conclude that LHY and CCA1 appear to be negative regulatory elements required for central oscillator function.

Introduction

Biological rhythms with a period close to 24 hr are called circadian rhythms and occur in many organisms. Such rhythms have been described extensively in mammals, insects, fungi, and plants (Dunlap, 1999; McClung, 2001). In plants, leaf movements, hypocotyl elongation, stomatal opening, floral induction, and approximately 6% of gene expression patterns can show circadian rhythms (reviewed by McClung [2001]). These rhythms are entrained by daily environmental cycles in light/dark or in temperature but can then be maintained under continuous conditions. Circadian rhythms enable daily changes in environmental conditions to be anticipated and, at least in bacteria, seem to provide a selective advantage (Ouyang et al., 1998).

The molecular basis of circadian rhythms has been intensively studied in *Drosophila*, *Neurospora*, mice, and cyanobacteria. In each of these organisms, a central oscillator based on feedback loops that regulate transcription has been proposed to generate the 24 hr periodicity of circadian rhythms (Dunlap, 1999). The oscillator responds to environmental conditions, particularly light and temperature, through input pathways that entrain it to the daily cycle. In turn, the oscillator is proposed to control many output pathways that each generate a rhythm in a particular phenotype or biochemical pathway.

Application of genetic and genomics approaches as well as noninvasive imaging technologies in the model plant species *Arabidopsis thaliana* has resulted in rapid progress in understanding the circadian system in plants (reviewed by McClung [2001]). The blue light photoreceptor Cryptochrome 1 (CRY) and the red/far-red photoreceptors Phytochrome A (PhyA), PhyB, PhyD, and PhyE mediate light input to the oscillator (Devlin and Kay, 2000; Somers et al., 1998a; Yanovsky et al., 2000). Mutations in the genes encoding these photoreceptors tend to lengthen the period of circadian rhythms, but their roles vary depending on the intensity and wavelength of light to which the plants are exposed. In addition, a number of other genes have been implicated in light input pathways. These include the related genes *ZEITLUPE* (*ZTL*) and *FKF1*, as well as *GIGANTEA* (*GI*) and *EARLY FLOWERING 3* (*ELF3*) (Somers et al., 2000; Nelson et al., 2000; Jarillo et al., 2001; Covington et al., 2001; Fowler et al., 1999; Liu et al., 2001; Park et al., 1999; McWatters et al., 2000).

Output pathways controlled by the circadian clock in plants have been described extensively by transcript profiling (Harmer et al., 2000; Schaffer et al., 2001). These approaches identified many circadian clock-regulated genes, which were then classified into functionally related groups involved in many metabolic and developmental processes (Harmer et al., 2000; Schaffer et al., 2001). One output pathway regulates flowering time via the *CONSTANS* (*CO*) gene (Suarez-Lopez et al., 2001). *CCR2* acts within another output pathway and encodes an RNA binding protein that regulates the translation of its own mRNA (Kreps and Simon, 1997; Heintzen et al., 1997). A complicating feature of the circadian system is that the expression of genes, such as *ELF3* and *GI*, that are implicated in input pathways are also circadian clock regulated, suggesting that some output pathways feedback to modulate input signaling to the oscillator (Liu et al., 2001; McWatters et al., 2000; Fowler et al., 1999; Park et al., 1999; Huq et al., 2000).

Three *Arabidopsis* genes that may encode components of the central oscillator are *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *TIMING OF CAB 1* (*TOC1*). LHY and CCA1 are closely related MYB-like transcription factors (Schaffer et al., 1998; Wang and Tobin, 1998). They are both circadian clock regulated with a peak in expression soon after dawn, and their overexpression causes arrhythmia in leaf movements, hypocotyl elongation, and many gene expression rhythms (Schaffer et al., 1998; Wang

[4]Correspondence: coupland@mpiz-koeln.mpg.de
[5]These authors contributed equally to this work.

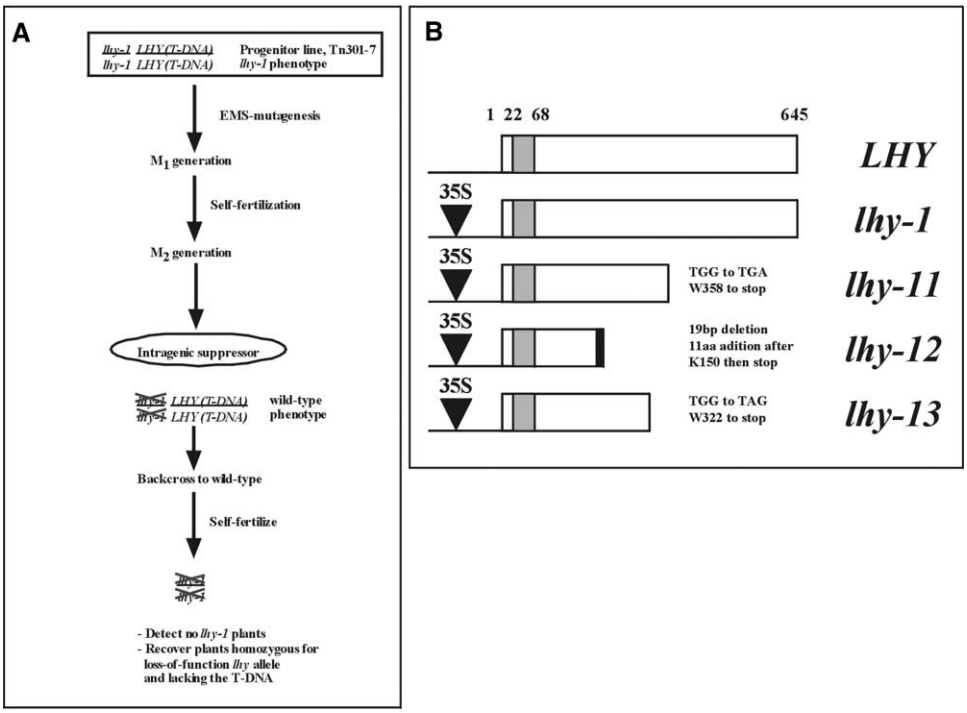

Figure 1. Isolation and Characterization of *lhy-1* Suppressor Mutations

(A) The mutagenesis procedure used to isolate *lhy-1* suppressor mutations. The progenitor line carried a wild-type transgenic copy of *LHY* as well as the dominant *lhy-1* mutation. The presence of the wild-type copy ensured that any M2 plant homozygous for an intragenic suppressor of *lhy-1* would have a wild-type phenotype.

(B) Mutations in *lhy-11*, *lhy-12*, and *lhy-13*.

and Tobin, 1998). A possible exception is *ELF3*, which was recently described to retain rhythmicity in plants overexpressing *LHY* (Hicks et al., 2001). Loss of *CCA1* function causes the circadian period to be shortened by around 3 hr, confirming that *CCA1* has a role in the circadian system but that it is not essential for circadian rhythms (Green and Tobin, 1999). *TOC1* encodes a protein containing a domain with similarity to the receiver domain of two-component response regulators but lacks residues essential for the function of typical response regulators (Strayer et al., 2000; Makino et al., 2000). In addition, *TOC1* contains a domain implicated in nuclear localization that is found near its carboxyl terminus and is also present in the CO protein, a regulator of flowering time. *TOC1* mRNA abundance peaks in the evening (Strayer et al., 2000; Makino et al., 2001), and mutations in the gene cause a short period phenotype. The effect of *toc1* on period length is independent of light intensity. The *toc1* mutation also alters the phase of circadian rhythms in plants entrained to temperature cycles, suggesting that *TOC1* is not simply involved in a light input pathway (Somers et al., 1998b). Recently, the *LHY/CCA1* and *TOC1* genes were proposed to be components of a feedback loop (Alabadi et al., 2001). LHY/CCA1 bind to the promoter of *TOC1* in vitro, and their overexpression reduces *TOC1* mRNA abundance (Alabadi et al., 2001). On the other hand, in *toc1* mutants, *LHY/CCA1* mRNA abundance cycles with a shorter period, and the *toc1-2* allele reduces the abundance of these mRNAs. Therefore, these three genes may define a transcriptional feedback loop (Alabadi et al., 2001; Schaffer et al., 1998; Wang and Tobin, 1998).

Here we describe the phenotype of *lhy* single and *lhy cca1* double loss-of-function mutants. Analysis of circadian rhythms in these plants indicates that the *LHY* and *CCA1* genes are partially redundant and that they are required for maintenance of circadian rhythms under constant conditions. Analysis of *TOC1* mRNA in these backgrounds and of *LHY/CCA1* mRNAs in *gi* mutants lead us to further elaborate models proposing a role for *LHY/CCA1* in the central oscillator of the plant circadian system.

Results

Isolation of *lhy* Loss-of-Function Alleles

The original *lhy-1* mutation is a dominant allele from which *LHY* mRNA is overexpressed due to the insertion of a transposon in the *LHY* 5′ untranslated leader (Schaffer et al., 1998). As well as disrupting circadian rhythms, the *lhy-1* mutation causes late flowering under long photoperiods and an elongated hypocotyl. To recover loss-of-function mutations in *LHY*, EMS mutagenesis was performed on *lhy-1* mutants, and M2 populations were screened for suppressors of *lhy-1*. Intragenic suppressors that inactivated the *LHY* gene were predicted to be a major class of the recovered mutations (Figure 1A and Experimental Procedures). In total, 117,600 M2 seedlings from four independent mutagenesis experiments were screened for individuals that had a shorter

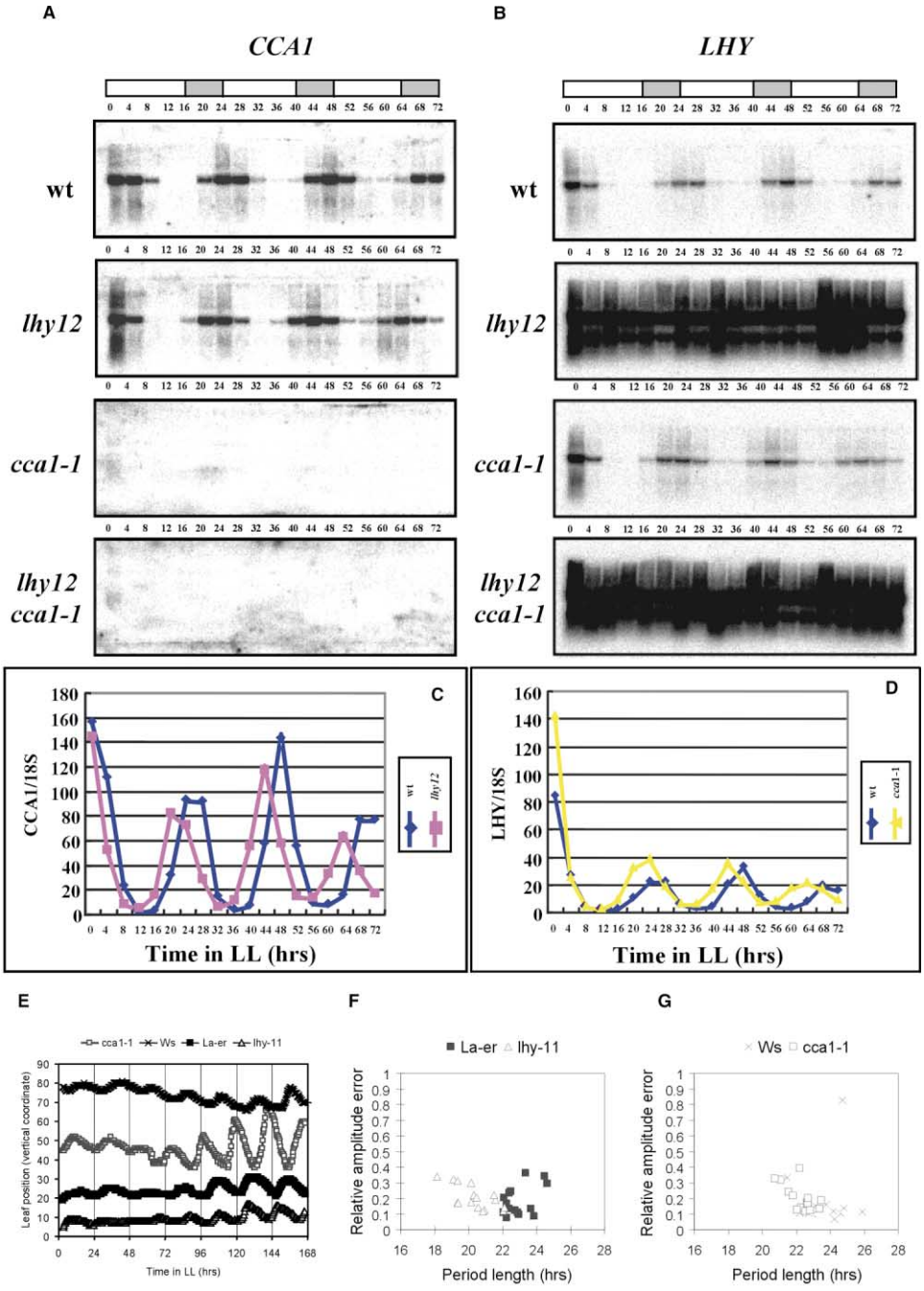

Figure 2. Analysis of *CCA1* and *LHY* mRNA and of Leaf Movements in *lhy-12* and *cca1-1* Mutants Grown under LL

Abundance of *CCA1* mRNA in L*er* and *lhy-12* (A, C, E, and F) and of *LHY* mRNA in L*er* and *cca1-1* (B and D). Plants were entrained to LD cycles (16L 8D) for 8 days and then released into LL (A, B, C, and D). Analysis is shown from the time just before transferring to LL (A–D). Blue, red, and yellow lines (C–H) represent L*er* wild-type, *lhy-12*, and *cca1-1*, respectively. Each experiment was performed at least twice with similar results. Quantification was performed on a Phosphorimager as described in Experimental Procedures. The effect of *lhy-12* and *cca1-1* on leaf movements is shown (G, H, and I) relative to appropriate wild-type controls. The results shown are combined data from two independent experiments. In each experiment, two leaves were analyzed from 32 plants (a total of 64 leaves). Period phenotypes were consistent between the two and were calculated as described in Experimental Procedures.

hypocotyl than *lhy-1* and appeared similar to wild-type seedlings (Figure 1A). Of eleven candidate mutants recovered in eight independent pools of M2 seedlings, three (EMS1-3C, EMS2-23A, and EMS4-64A) were studied in detail. These plants were self-fertilized, and the M3 progeny were confirmed to carry the transposon in the 5′ untranslated leader of the *LHY* gene by PCR (Experimental Procedures). Therefore, the three M2 seedlings that exhibited a wild-type hypocotyl were derived from *lhy-1*. The flowering time of the M3 plants

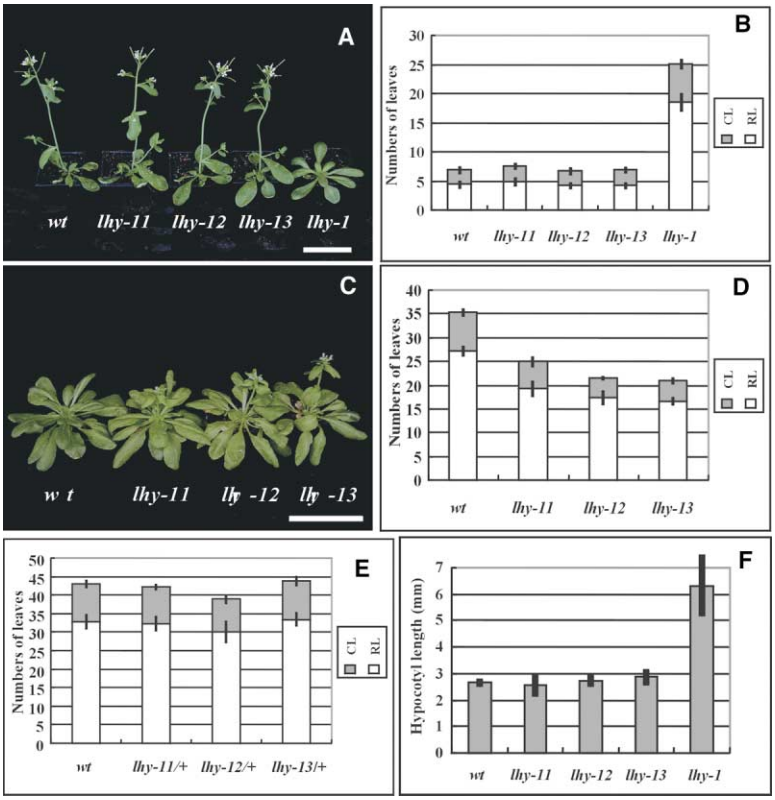

Figure 3. Early Flowering Phenotype of the *lhy* Loss-of-Function Mutants

Plants were grown for 4 weeks under long days (A and B) or for 7 weeks under short days (C and D). Flowering time under short days of the F1 generation of the *lhy* loss-of-function alleles crossed to L*er* (E). Means are shown ± standard deviation. Open and dark boxes represent numbers of rosette and cauline leaves, respectively. The length of the hypocotyls of wild-type, *lhy* loss-of-function mutants and *lhy-1* grown under 16L 8D (F).

was identical to that of wild-type plants under long days, demonstrating that this aspect of the *lhy-1* phenotype was also suppressed in these lines.

To study the inheritance of the putative suppressor mutations, M3 or M4 plants derived from each of the three lines were backcrossed to wild-type plants (Figure 1A and Experimental Procedures). All the F1 plants had short hypocotyls, indicating that the *lhy-1* phenotype was not restored in the F1 and, therefore, that the suppressor mutations were either intragenic or extragenic and dominant. The F1 plants were self-fertilized, and the hypocotyls of the F2 seedlings were examined. None of the F2 plants exhibited the elongated hypocotyl characteristic of *lhy-1*, excluding the possibility that the suppressor mutations were extragenic, unless they were dominant and tightly linked to *lhy-1*. The mutations present in lines EMS1-3C, EMS2-23A, and EMS4-64A were preliminarily assigned the names *lhy-11*, *lhy-12*, and *lhy-13*, respectively.

To confirm that these mutations were intragenic suppressors and to characterize their effects on *lhy-1*, *LHY* cDNAs were isolated from each line, and their sequences were obtained. Comparison of these sequences to that of the *LHY* cDNA from Landsberg *erecta* (L*er*) identified a change from G to A at +1414 (W to stop codon) in *lhy-11*, a 19 bp deletion from +782 to +801 in *lhy-12*, and a change from G to A at +1302 (W to stop codon) in *lhy-13*. Identification of the mutations within the *LHY* coding sequence confirmed that *lhy-11*, *lhy-12*, and *lhy-13* are intragenic suppressor mutations of *lhy-1* (Figure 1B) and demonstrated that they carry mutations predicted to severely impair or abolish *LHY* function.

lhy Loss-of-Function Alleles Shorten the Period of Circadian Rhythms

Loss-of-function mutations in *CCA1* cause circadian rhythms to cycle with a shorter period (Green and Tobin, 1999). Therefore, the effect of the *lhy* loss-of-function mutations on circadian rhythms was tested. To test the period length of free-running rhythms (FRRs), *lhy-12* seedlings were grown under light/dark (L/D) cycles and then shifted to continuous light (LL; Experimental Procedures). Wild-type plants and *cca1-1* mutants were used as controls. Seedlings of each genotype were harvested at dawn (Zeitgeber time [ZT] 0 [hours]; Experimental Procedures) and then every 4 hr for 72 hr under continuous conditions. RNA gel blots were made and hybridized with a *CCA1* or *LHY* gene probe (Figure 2 and Experimental Procedures).

Under LL, in wild-type plants, a circadian rhythm in *CCA1* mRNA abundance occurred with peaks in abundance at 0 hr, 24 hr, 48 hr, and 72 hr, but an insertion prevented expression of the *CCA1* mRNA in *cca1-1* (Figures 2A and 2C; Green and Tobin, 1999). In *lhy-12*, circadian rhythms in *CCA1* mRNA abundance were still detected, but the peaks in the abundance of the mRNA occurred earlier than in wild-type plants (Figures 2A and 2C). Analysis of these patterns indicated that, in *lhy-12*, rhythms in *CCA1* mRNA abundance showed a period 2–3 hr shorter than in wild-type plants (Experimental Procedures). Similar results were obtained for several other circadian clock-controlled genes (CCGs; see later) and for *CCA1* expression in the *lhy-11* and *lhy-13* mutants (data not shown).

The effect of *lhy-11* on the circadian rhythm of leaf

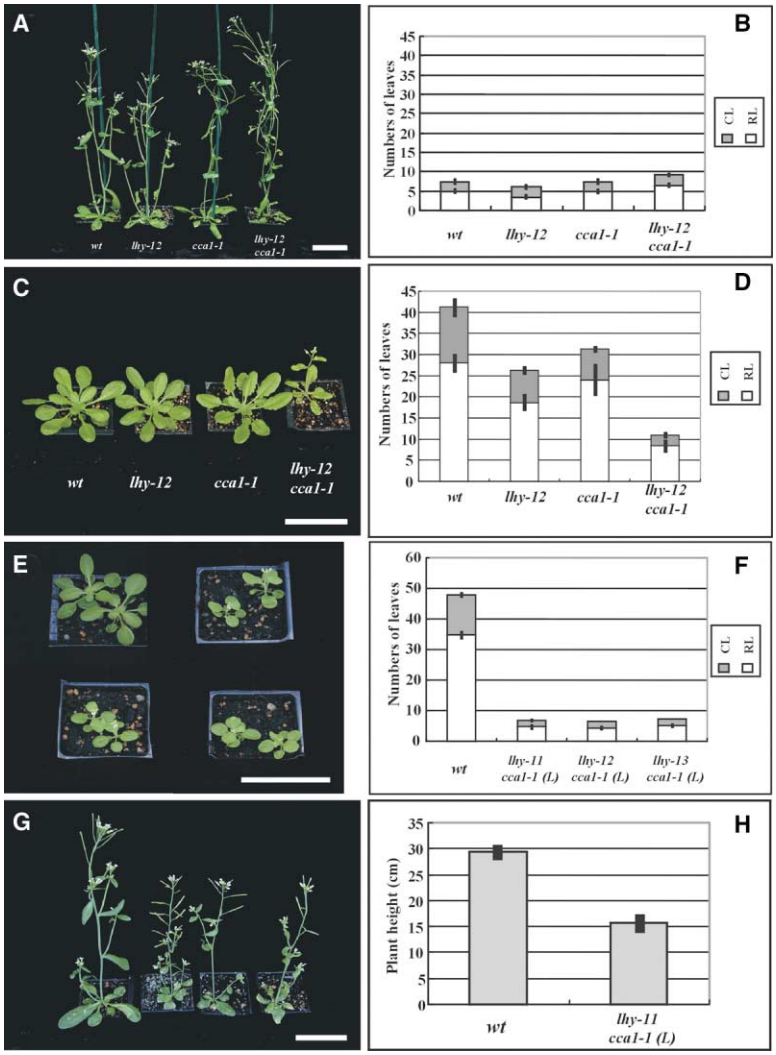

Figure 4. Phenotypes of the *lhy cca1-1* Double Loss-of-Function Mutants

Flowering times of L*er*, *lhy-12*, *cca1-1*, and *lhy-12 cca1-1* under LD (A and B) and SD (C and D).

(E) Early flowering and pale-green phenotypes of the *lhy cca1-1* double nulls under SD: top left, L*er*; top right, *lhy-11 cca1-1* (BC4); bottom left, *lhy-12 cca1-1* (BC4), and bottom right, *lhy-13 cca1-1* (BC5).

(F) Analysis of the flowering times of the plants shown in (E).

(G and H) Reduced height of the *lhy cca1-1* plants under LD.

(B, D, and F) Mean numbers of rosette (open boxes) and cauline leaves (dark boxes) ± standard deviation are shown.

(H) Mean height (cm) ± standard deviation are indicated.

Plants were grown under the same conditions as those in Figure 3. The LD-grown plants shown were 4 weeks old (A, B, G, and H), and the SD-grown plants were 3 weeks old (C, D, E, and F).

movements was also tested in entrained plants shifted to LL. The *lhy-11* mutant exhibited rhythmic leaf movements (Figures 2G and 2H). Statistical analysis of leaf movement data (Experimental Procedures) showed that the rhythms obtained were as robust as those of the wild-type parent (L*er*), as indicated by the narrow range of periods extracted and their low relative amplitude error values (Figure 2H). However, the period of the rhythm in *lhy-11* was approximately 2.5 hr shorter than in wild-type plants (Figures 2G and 2H). Similar results were obtained for *cca1-1* (Figures 2G and 2I). The period of the leaf movement rhythm in *cca1-1* plants was 2.5 hr longer than in *lhy-11* plants, but this probably reflects differences between parental ecotypes, since the period of the rhythm in Ws was approximately 2 hr longer than in L*er* (Figures 2H and 2I).

LHY mRNA abundance showed a similar pattern to that of *CCA1* in wild-type plants under LL and in *cca1-1* mutants cycled with a short period, as previously demonstrated (Figures 2B and 2D; Schaffer et al., 1998; Green and Tobin, 1999). In *lhy-12* mutants *LHY* mRNA was present at a constantly high level (Figure 2B), confirming that *lhy-12* was derived from the dominant *lhy-1* mutation.

The Effects of *lhy* Loss-of-Function Alleles on Flowering Time and Hypocotyl Length

The *toc1* mutation causes a short period phenotype similar to that of *lhy-12*, and this is associated with early flowering under short photoperiods (Millar et al., 1995a; Somers et al., 1998b). Therefore, the flowering time of *lhy-11*, *lhy-12*, and *lhy-13* plants was measured under long and short days (LD and SD) in controlled environment cabinets. Wild-type and gain-of-function *lhy-1* plants were used as controls. Under LDs, *lhy*-11, *lhy-12*, and *lhy-13* mutants flowered at a similar time to wild-type plants, whereas the *lhy-1* mutant was later flowering (Figures 3A and 3B). However, under SDs, all three *lhy* loss-of-function mutants flowered earlier than wild-type plants (Figures 3C and 3D), and application of the Student's t test confirmed that these differences were statistically significant. Plants heterozygous for the *lhy* loss-of-function mutations flowered with a similar number of leaves to wild-type plants (Figure 3E), and, therefore, these mutations are recessive.

The hypocotyls of *lhy-11*, *lhy-12*, and *lhy-13* mutants were also compared with those of wild-type plants and *lhy-1* mutants. The three genotypes were grown under L/D cycles of white light and darkness. The *lhy-1* mutant

has an elongated hypocotyl under these conditions (Figure 3F; Schaffer et al., 1998), whereas the hypocotyls of the *lhy* loss-of-function mutants were similar to those of wild-type plants (Figure 3F).

cca1-1 Enhances the Early Flowering Phenotype of *lhy* Loss-of-Function Mutants, and the Double Mutant Is Reduced in Stature

The loss-of-function *lhy* mutations cause early flowering under short days (Figures 3C and 3D). *CCA1* is closely related to *LHY* in sequence and function (Schaffer et al., 1998; Wang and Tobin, 1998), but the flowering time of *cca1-1* mutants was not previously reported (Green and Tobin, 1999). After two backcrosses to L*er* (Experimental Procedures), the *cca1-1* mutant was scored for flowering time and shown to flower at a similar time to wild-type under LD (Figures 4A and 4B) but earlier than wild-type under SD (Figures 4C and 4D). Application of the Student's t test confirmed that this difference was statistically significant. To test whether the effects of *lhy* and *cca1-1* on flowering time are additive or whether one mutation is epistatic to the other, *lhy-12 cca1-1* double mutants were constructed. Under SD, the double mutant flowered much earlier than wild-type plants or the progenitor mutants (Figures 4C and 4D) but, under LD, was similar to wild-type (Figures 4A and 4B). Mutations that impair the function of *CCA1* and *LHY* therefore have additive effects on flowering time under SD, consistent with the genes having partially redundant roles in controlling flowering time.

The early flowering phenotype of *lhy-11 cca1-1*, *lhy-12 cca1-1*, and *lhy-13 cca1-1* double mutants was even more pronounced after 2–3 more backcrosses to L*er* (Figures 4E and 4F; Experimental Procedures). These plants were also consistently smaller than the wild-type plants and the progenitor mutants. The rosette leaves of the double mutants were smaller and paler than those of wild-type plants (Figure 4E), and the main stem was shorter (Figures 4G and 4H). This reduction in size occurred under long and short days.

Effects of Loss of *LHY* and *CCA1* Function on Leaf Movement Rhythms

Whether the short circadian period phenotype of *lhy* mutants (Figure 2) was also enhanced by *cca1-1* was tested. Leaf movements were recorded in entrained plants shifted to LL (Experimental Procedures). Visual observation of time-lapse images and of individual leaf movement traces (Figure 5E) suggested that *lhy-11 cca1-1* double mutants did not move their leaves in a rhythmic manner. This assessment was confirmed by statistical analysis of the data (Figure 5F). Many period values extracted for double mutants were associated with high relative amplitude errors (above 0.4), suggesting that rhythmic signals identified in the double mutant were weaker than in wild-type and single mutant plants. Furthermore, period values varied widely between data sets and ranged between 45 and 96 hr. This lack of period consensus suggests that the timing mechanism that underlies leaf movement rhythms in wild-type plants was disrupted in the *lhy-11 cca1-1* double mutant.

Effects of Loss of *LHY* and *CCA1* Function on Circadian Clock-Controlled Gene Expression Rhythms that Peak in the Morning

To analyze expression of CCGs, *lhy-12 cca1-1* seedlings, progenitor single mutants, and wild-type plants were entrained under L/D conditions and then shifted to LL (Experimental Procedures). At ZT 0 and at 4 hr intervals for 72 hr, seedlings were harvested, and RNA was extracted. First, genes that peak in expression soon after dawn, and therefore show a similar phase of expression to *LHY* and *CCA1*, were tested. Expression of *LHY* and *CCA1* cannot be monitored because they contain insertions (Figure 2). Therefore, the patterns of expression of the *CO-Like 1* (*COL1*; Ledger et al., 2001) and *At5g17300* (Arabidopsis Genome Initiative, 2000) genes were tested. In wild-type plants both genes peak in expression around subjective dawn at ZT 24, ZT 48, and ZT 68 (Figures 5A and 5C). In each single mutant the period of the rhythm in *COL1* and *At5g17300* mRNA abundance is shorter by approximately 2–3 hr, which is consistent with the analysis of expression of other CCGs in *lhy-12* (Figures 2, 5A, and 5C) and *cca1-1* mutants (Figures 2, 5A, and 5C; Green and Tobin, 1999). In the *lhy-12 cca1-1* double mutant, *COL1* mRNA and *At5g17300* mRNA peak in abundance at ZT 0, but they fall to trough levels by ZT 4, whereas, in wild-type plants or the single mutants, they do not reach trough levels until ZT 8 (Figures 5B and 5D). *At5g17300* mRNA abundance rises again in the double mutant around ZT 16 but does not show any subsequent, regular oscillations of high amplitude (Figure 5D). The double mutant, therefore, does not show detectable, overt circadian rhythms in *At5g17300* mRNA abundance after 24 hr in LL. *COL1* mRNA abundance peaks at around ZT 20 and ZT 36 (Figure 5B) in *lhy-12 cca1-1* mutants, so that the latter peak is around 12 hr earlier than the corresponding peak in wild-type plants (Figure 5A). This suggests that the period of the FRR in *COL1* expression is approximately 6 hr shorter in the double mutant than in wild-type plants. Subsequently, the rhythm in *COL1* expression dampens in the double mutant, and, in contrast to the single mutants, a rhythm in expression is not apparent after 40 hr in LL (Figures 5A and 5B). Thus, under LL, loss of function of both *LHY* and *CCA1* causes dramatic shortening of the period of circadian rhythms in *COL1* and *At5g17300* expression and rapid dampening of these rhythms.

Effect of Loss of *LHY* and *CCA1* Function on Circadian Clock-Controlled Gene Expression Rhythms that Peak in the Evening

LHY and *CCA1* are required for sustained circadian rhythms in genes that cycle in a similar phase to themselves (Figures 2 and 5). Whether the *GI*, *TOC1*, and *CCR2* genes that peak in the evening (Carpenter et al., 1994; Park et al., 1999; Fowler et al., 1999; Strayer et al., 2000; Makino et al., 2001) are similarly affected in the *lhy cca1-1* double mutant was also tested. Their expression was analyzed in wild-type, *lhy-12*, *cca1-1*, and *lhy-12 cca1-1* plants entrained to L/D and then shifted to LL. In wild-type plants these genes showed the expected patterns of expression with peaks at approximately ZT 12 (ZT 8 for *GI* in this experiment), ZT

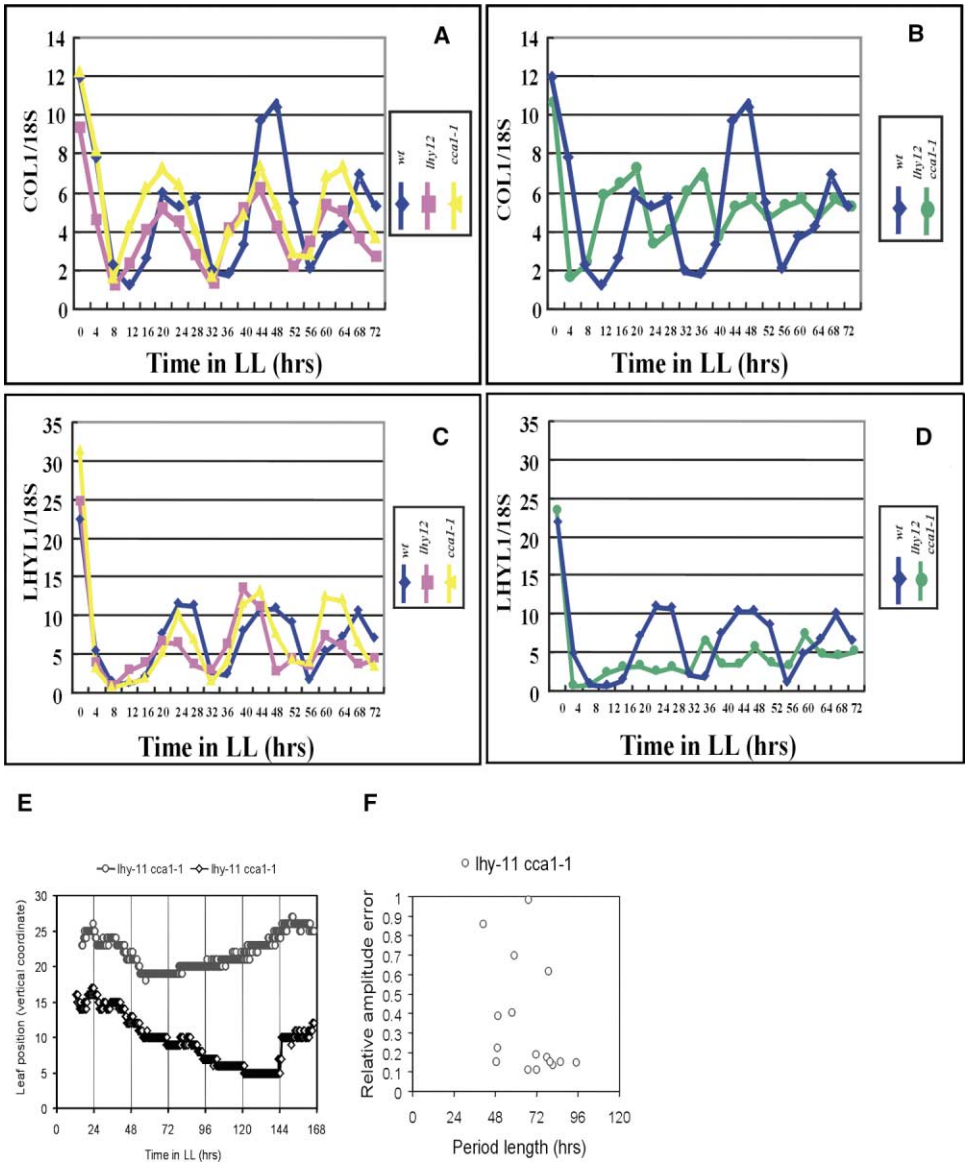

Figure 5. Expression of the Morning-Phased Genes and Analysis of Leaf Movements in the *lhy cca1-1* Double Null Mutant under LL

Abundance of *COL1* mRNA (A and B) and *At5g17300* mRNA (labeled *LHYL1*; [C and D]) under LL. Blue, red, yellow, and green lines represent L*er*, *lhy-12*, *cca1-1*, and *lhy-12 cca1-1*, respectively. Plants were treated, and data is shown as described in Figure 2. Each experiment was performed at least twice with similar results. Quantification was as described in Figure 2. The effects of *lhy-12 cca1-1* on leaf movements under LL are shown (E and F), and the data are taken from independent experiments.

36, and ZT 60 (Figures 6A, 6C, and 6E). In the *lhy-12* and *cca1-1* single mutants, all three genes cycle with a short period, so that, under LL, the third peak in expression of each gene occurs at approximately ZT 52 (Figures 6A, 6C, and 6E). In the *lhy-12 cca1-1* double mutants, two clear peaks in mRNA abundance occur at ZT 4 and ZT 24 for each of the three genes (Figures 6B, 6D, and 6F). The latter is approximately 12 hr earlier than the corresponding peak in wild-type plants. In contrast to wild-type or single mutant plants, no rhythms in expression of the three genes were detected in the double mutant after ZT 40 (Figure 6A–6F). Thus, in the *lhy-12 cca1-1* double mutant, genes that peak in expression in the evening cycle with a period approximately 6 hr shorter than wild-type, and their oscillations dampen much more rapidly (Figures 6A–6F). The period shortening and dampening of FRRs in genes that peak in expression in the evening are similar to those described for genes with a morning phase of expression (Figures 5A–5E).

The Phase of Expression of Genes Expressed in the Evening Is Shifted Earlier in *lhy cca1-1* Double Mutants under L/D Cycles

The shift in timing of the peak in expression of evening-phased CCGs was observed in the first peak in expression under LL (Figures 6A–6F). This shift in phase in LL

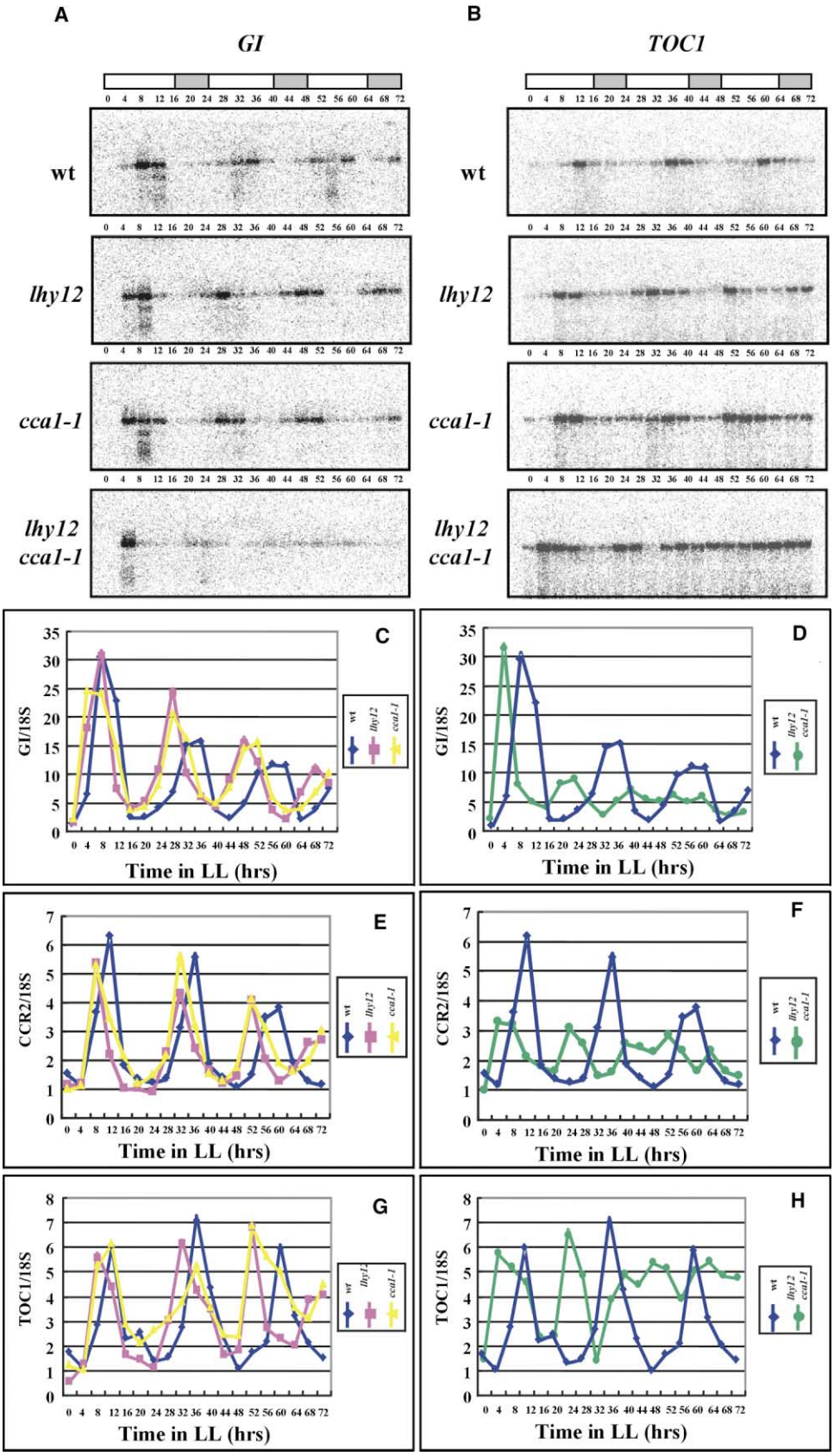

Figure 6. Expression of Evening-Phased Genes in the *lhy cca1-1* Double Mutant under LL

Abundance of the mRNAs of three evening-phased genes, *GI* (A and B), *CCR2* (C and D), and *TOC1* (E and F). Blue, red, yellow, and green lines represent L*er*, *lhy-12*, *cca1-1*, and *lhy-12 cca1-1*, respectively. Plants were treated, and data is shown as described in Figure 2. Each experiment was repeated at least twice with similar results. Quantification as in Figure 2.

soon after the plants are moved from darkness suggested that the expression of evening genes might be strongly phase shifted in plants grown under daily L/D cycles. To test this, *GI* and *TOC1* mRNA abundance was examined in *lhy-12 cca1-1* double mutants, the single mutant progenitors, and wild-type plants grown under L/D cycles. The peaks in *GI* and *TOC1* mRNA abundance occurred approximately 6 hr earlier in the *lhy-12 cca1-1* double mutant than in wild-type plants under these conditions (Figures 7A–7F). The amplitude of the rhythm in *TOC1* expression is also reduced, because trough levels of *TOC1* mRNA are higher (Figures 7B and 7F). Peak levels of the *COL1* and *At5g17300* mRNAs that peak in expression in the morning are not phase shifted in the double mutant plants under similar conditions (Figures 7G–7J).

The *gi-3* Mutation Causes Rapid Dampening of *LHY* and *CCA1* Expression under LL

In the *lhy-12 cca1-1* double mutants, the phase of expression of *GI* and *TOC1* was shifted earlier under L/D cycles (Figure 7). Previously, a reduction in expression of *LHY* and *CCA1* was reported in *gi-3* mutants under L/D (Fowler et al., 1999), in the Columbia *gi-1* and *gi-2* mutants under LL (Park et al., 1999), and in *toc1-2* mutants under LL (Alabadi et al., 2001). The effect of the *gi-3* mutation on *LHY* and *CCA1* expression was tested under LL conditions (Figures 7K and 7L). The level of expression of *LHY* and *CCA1* was reduced at ZT 0 in *gi-3* mutants, and the amplitude of subsequent free-running rhythms was dramatically lower. *GI* is therefore required for high-amplitude circadian rhythms in *LHY* and *CCA1* expression under these conditions.

Discussion

Novel loss-of-function *lhy* mutations were used to study the role of *LHY* in the circadian system of *Arabidopsis* and to describe the relationship between *LHY* and the related gene *CCA1*. Three of these mutations cause similar phenotypes and are each predicted to truncate the LHY protein to less than half of its original size, consistent with the idea that they severely impair or abolish LHY activity. As expected, all are recessive, at least with respect to their flowering time phenotype. Analysis of these new *lhy* mutations and their combination with *cca1-1* loss-of-function mutations enabled us to demonstrate that these genes are required for the maintenance of free-running rhythms (FRRs) in leaf movements and in CCG expression under LL, and this is discussed in more detail in the following sections.

Interpretation of the Genetic Redundancy between *LHY* and *CCA1*

The *lhy cca1-1* double mutant shows more dramatic phenotypes than either single mutant. For example, the double mutant exhibits an enhanced short period phenotype under LL, rapid dampening of rhythms under LL, a shift in the phase of evening expressed genes under L/D, and an enhanced early-flowering phenotype. However, the phenotypes of the single mutants demonstrate that *LHY* and *CCA1* do not have completely overlapping functions (Figures 2 and 3; Green and Tobin, 1999). Similar genetic redundancy in which the effect of mutations in a single gene is strongly enhanced by a second mutation in a related gene has been described in the mammalian circadian system in the families of *mPer* and *mCry* genes (reviewed by Wager-Smith and Kay [2000]). Genetic redundancy between the *lhy* and *cca1-1* mutations could be explained in two ways. First, they might have distinct roles in the control of circadian rhythms, so that disruption of both functions has a strongly additive effect, resulting in the enhanced phenotype of the double mutant. Such a relationship has been proposed for the *mPer1* and *mPer2* genes in the control of circadian rhythms (Zheng et al., 2001). Mutations in each of these genes caused a short period phenotype, and the double loss-of-function mutant was arrhythmic, but the roles of the two genes within the circadian system are different (Zheng et al., 2001). Alternatively, *LHY* and *CCA1* may have identical or almost identical functions. In this case, the mutant phenotypes of the single mutants would be due to the genes having both common and unique functions or to them having identical functions, but the dosage of the combined gene products would be crucial for their function. That *LHY* and *CCA1* have identical or almost identical functions is the most parsimonious explanation for the redundancy between *lhy* and *cca1-1*. This explanation is also consistent with the observations that the single loss-of-function mutants have apparently identical phenotypes (Figures 2 and 3; Green and Tobin, 1999), that overexpression of each gene appears to have identical effects (Schaffer et al., 1998; Wang and Tobin, 1998), that both *LHY* and *CCA1* are expressed in the same phase, and that the expression of both mRNAs responds strongly to light in etiolated plants (Schaffer et al., 1998; Wang and Tobin, 1998; Martinez-Garcia et al., 2000). Thus, *LHY* and *CCA1* probably have identical or very closely related functions within the circadian system, and the enhanced phenotype of the double mutant is likely to be due to each protein largely compensating for loss of the other in the single mutants.

The Pleiotropic Phenotypes of *lhy cca1-1* Double Mutants

In addition to the effect on circadian rhythms, the *lhy cca1-1* double mutant showed dramatic early flowering under short days and reduced stature. Circadian rhythms are closely associated with the photoperiodic control of flowering (Samach and Coupland, 2000), and several mutations that disrupt the circadian system of *Arabidopsis* also affect flowering time (Hicks et al., 1996; Schaffer et al., 1998; Somers et al., 1998b, 2000; Nelson et al., 2000; Fowler et al., 1999; Park et al., 1999). This is often explained in terms of an external coincidence model, which proposes that a light-sensitive phase of a circadian rhythm is exposed to light in inductive conditions but not in noninductive conditions, and this regulates flowering time (reviewed by Samach and Coupland [2000]). The circadian clock-controlled *CONSTANS* gene that regulates flowering time of *Arabidopsis* was recently proposed to mediate between the circadian system and the control of flowering (Suarez-Lopez et al., 2001). Disruption of the circadian system in the *lhy cca1-1* double mutant may therefore underlie its flowering time phenotype. In particular, the shift in phase of

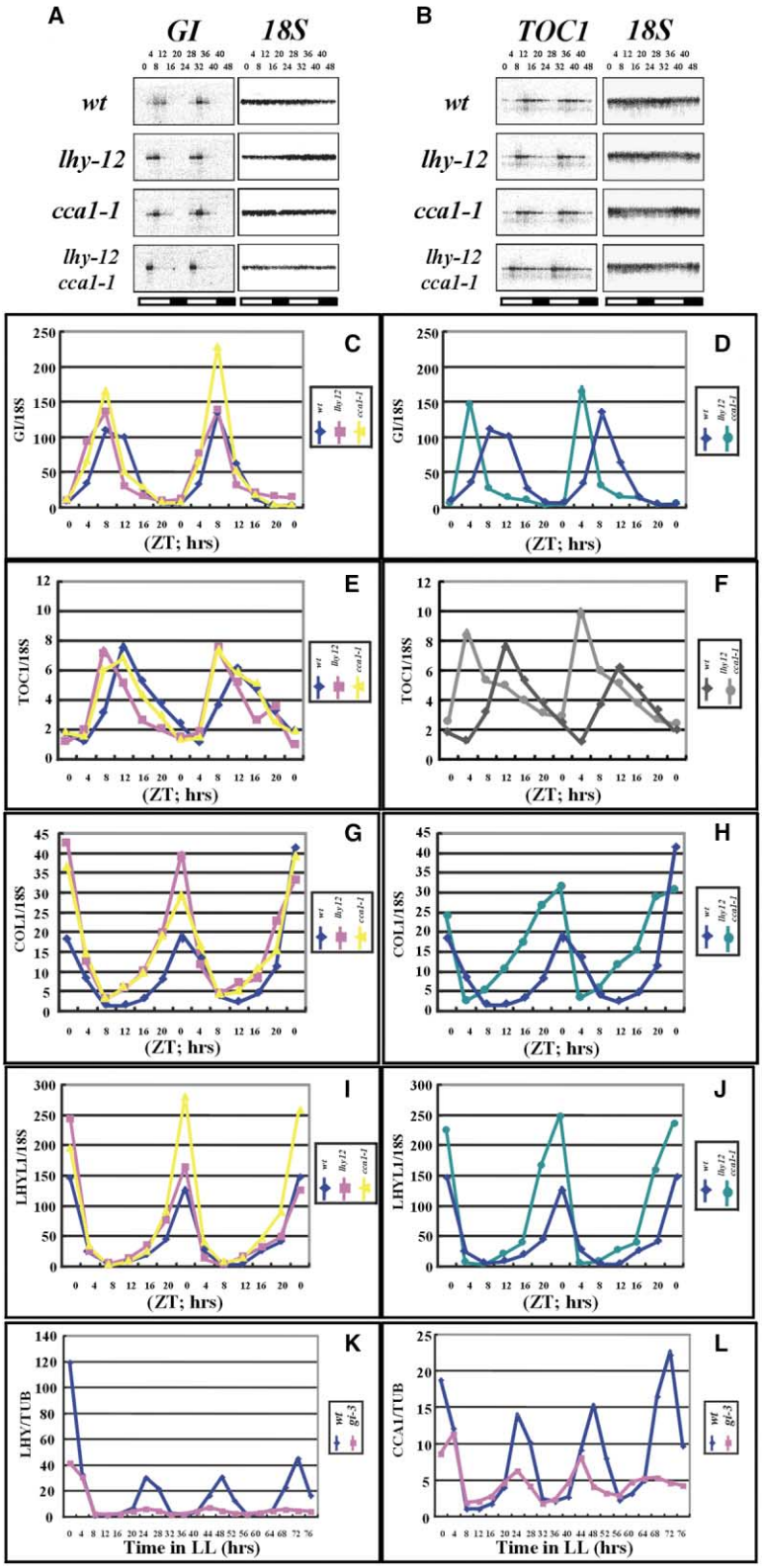

Figure 7. Expression of CCGs under Light/Dark Cycles and the Effect of Inactivation of *GI* on *LHY*/CCA1 Expression

The abundance of the mRNAs of two evening-phased genes, *GI* (A, C, and D), and *TOC1* (B, E, and F), and two morning-phased genes, *COL1* (I and J) and *At5g17300* (labeled *LHYL1;* [K and L]), under light/dark cycles. Blue, red, yellow, and green lines represent L*er*, *lhy-12*, *cca1-1*, and *lhy-12 cca1-1*, respectively. Plants were grown under LD condition (16L 8D) for 8 days. Zeitgeber Time (ZT) 0 indicates the time point just before lights on. Each experiment was repeated at least twice with similar results. The effect of the *gi-3* mutation on *LHY* (K) and *CCA1* (L) mRNA abundance under LL. Wild-type, blue; *gi-3*, red. Plants were treated and quantifications performed as described in Figure 2.

circadian rhythms that occurs in these plants under L/D may cause the light-sensitive phase of a rhythm that controls flowering to occur in the light in short days, whereas, in wild-type plants, it would occur in darkness. Such a model could explain the earlier flowering of double mutant plants under short days.

The effect of the *lhy cca1-1* double mutant on plant stature was not expected. No other mutations within

the circadian system of *Arabidopsis* were previously reported to have such an effect. Nevertheless, the effect of *lhy cca1-1* on circadian clock-controlled gene expression under standard L/D growth conditions is much stronger than has been reported for these other mutant genotypes. Also, since it is now clear that many hundreds of *Arabidopsis* genes are circadian clock regulated (Harmer et al., 2000; Schaffer et al., 2001), such an alteration in their expression may be expected to have effects on plant growth.

lhy cca1-1 Double Mutants Show Diurnal Rhythms under Light/Dark Cycles

As described previously, the *lhy cca1-1* double mutant becomes arrhythmic for leaf movements and CCG expression rhythms after several cycles under LL, but, under L/D, rhythms in GI, TOC1, COL1, and *At5g17300* are retained. Direct activation of transcription of these genes by transition from dark to light could generate such rhythms. An alternative possibility is that transitions from light to dark induce the expression of a gene that can compensate for the function of *LHY* and *CCA1* in the circadian system. These L/D-driven rhythms may then continue under constant conditions for one or two cycles without *LHY* and *CCA1* but cannot be maintained under these conditions.

A feature of the diurnal rhythms detected in *lhy-11 cca1-1* plants under L/D cycles is that their phase can differ strikingly from those detected in wild-type plants. This is most apparent for genes that peak in the evening, such as *GI*, *TOC1*, and *CCR2*. Therefore, although *LHY* and *CCA1* normally peak in expression at or soon after dawn, the most dramatic effect under L/D of inactivation of these genes is to cause much earlier expression of genes that normally peak in the evening (Figure 7). Therefore, *LHY* and *CCA1* may normally act in the morning to prevent premature expression of evening genes. Such an effect may be indirect, for example, by affecting entrainment of the oscillator and thereby altering the phase of gene expression rhythms. However, this observation is also consistent with a direct role for LHY and CCA1 in the repression of evening genes, and this is supported by the presence of several copies of the evening element, a motif closely related to the LHY/CCA1 binding site, in the promoters of *GI*, *TOC1*, and *CCR2* (Wang et al., 1997; Harmer et al., 2000). Furthermore, LHY and CCA1 will bind in vitro to copies of this motif present in the *TOC1* promoter (Alabadi et al., 2001).

Despite these similarities in the structure and expression of the *GI* and *TOC1* genes, our analysis also revealed striking differences in their expression in the *lhy cca1-1* double mutant. After around ZT 40 in LL, when oscillations in *GI* and *TOC1* are no longer detectable, *TOC1* mRNA abundance becomes arrhythmic at high expression level, whereas *GI* is arrhythmic at low expression level. This is consistent with the idea that LHY and CCA1 may act to repress *TOC1* expression, and that *TOC1* is reduced in expression in *LHY*-overexpressing plants (Alabadi et al., 2001). However, the low level of expression of *GI* in *lhy cca1-1* plants suggests a more complex pattern of regulation, in which high levels of *GI* expression also require proteins that are absent in an *lhy cca1-1* double mutant, perhaps as an indirect effect of the lack of circadian rhythms in these plants.

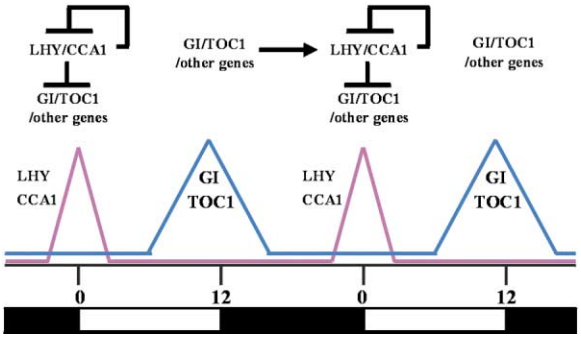

Figure 8. A Schematic Model for the Role of *LHY/CCA1* in the Circadian System of *Arabidopsis*

LHY/CCA1 expression rises just prior to lights on and reaches peak levels soon after dawn. The LHY/CCA1 proteins are proposed to repress *GI* and *TOC1* expression (Figures 6,7; Alabadi et al., 2001) and feedback to repress their own expression (Schaffer et al., 1998, Wang and Tobin, 1998). As the expression of *LHY* and *CCA1* subsides, the levels of *GI* and *TOC1* mRNAs rise, reaching peak levels 10–12 hr after dawn. The *GI* and *TOC1* genes promote expression of *LHY* and *CCA1*, thus starting another cycle of regulation.

A Possible Role for *LHY* and *CCA1* in the Central Oscillator of the Plant Circadian System

Our data provide strong genetic support for previous proposals that *LHY* and *CCA1* may be components of the central oscillator of the plant circadian system. Major points are as follows: (1) in the absence of the LHY and CCA1 proteins, circadian rhythms dampen rapidly in LL (Figure 2), (2) constant overexpression of *LHY* or CCA1 causes arrhythmicity in LL and DD and represses expression of both genes (Schaffer et al., 1998; Wang and Tobin, 1998), (3) the expression of *LHY* and *CCA1* is rhythmic, and (4) period length may be affected by dosage of the *LHY/CCA1* gene products in *lhy* or *cca1-1* single mutants. LHY and CCA1 may therefore be components of the central oscillator. This is more likely than these proteins regulating light input to the oscillator, which would not easily explain the phenotypes caused by *LHY* overexpression in DD (Schaffer et al., 1998). Similarly, they are less likely to act as part of an output pathway, because this would not readily explain the effects of gain- and loss-of-function mutations on many rhythms expressed in different phases.

The oscillators of model animal and bacterial systems such as *Drosophila*, *Neurospora,* mouse, and cyanobacteria are negative feedback loops that act at the level of transcription-translation. Schematically, these loops are composed of positive elements that drive the transcription of negative elements, which, in turn, feed back to repress their own transcription (Dunlap, 1999). As the levels of negative elements fall, the positive elements can again activate their transcription. Our data and those of Alabadi et al. (2001) provide evidence for the involvement of *LHY* and CCA1 in such a cycle. *LHY* and *CCA1* transcription rises early in the day, which may reflect a direct response to light as well as circadian regulation (Martinez-Garcia et al., 2000; Wang and Tobin, 1998). The model proposes that these genes then act as negative elements that repress the expression of positive elements (Figure 8). As *LHY* and CCA1 expression subsides, positive elements rise and eventually peak in the

evening. Alabadi et al. (2001) argued that TOC1 was such a positive element because the *toc1-2* mutation caused a reduction in *LHY/CCA1* expression. We show here that *gi-3* mutations have an equally dramatic effect on the amplitude of *LHY/CCA1* transcription under LL, consistent with previous observations that *LHY* expression was reduced under L/D in *gi-3* mutants and under LL in *gi* alleles recovered in another *Arabidopsis* ecotype (Fowler et al., 1999; Park et al., 1999). Mutations in *GI* can also have general effects on the period length of circadian rhythms that vary with the allele studied (Park et al., 1999). Therefore, there may be multiple positive elements that normally peak in a similar evening phase and, in wild-type plants, are restricted to this phase by LHY and CCA1 (Figure 8).

Experimental Procedures

Plant Materials and Growth Conditions

The L*er* ecotype of *Arabidopsis thaliana* was used unless otherwise indicated. The *lhy-1* (originally named *lhy*) and *cca1-1* mutants were described previously (Schaffer et al., 1998; Green and Tobin, 1999). The *cca1-1* mutant was originally in the Ws background, whereas *lhy-1* was in L*er*. F3 populations introgressed twice into L*er* were selected for *cca1-1* homozygous lines based on Km resistance (progeny of the first back cross were a gift of R. Green and E. Tobin, UCLA). *lhy-11 cca1-1*, *lhy-12 cca1-1*, and *lhy-13 cca1-1* double null mutants were obtained as described in the following section. Plants were grown on soil in controlled environment rooms under either LD (10 hr light/6 hr day extension/8 hr dark) or SD cycles of 10 hr light and 14 hr dark (10L 14D) as described (Putterill et al., 1995). For LL and DD experiments, plants were grown on agar plates under LD (16 L 8D) for 8 days and then transferred to LL and DD at dawn (Schaffer et al., 1998).

Measurement of Flowering Time

Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem. Data are presented as mean ± SEM. Student's t test was used to determine whether differences were statistically significant. All differences in flowering time were confirmed as statistically significant using the t test ($p < 0.00005$).

Isolation of Loss-of-Function Alleles of *lhy* and Construction of Introgressed *cca1 lhy* Double Mutants

To identify a loss-of-function *lhy* allele, an *lhy-1* mutant line into which a wild-type copy of the gene had been introduced by transformation (line Tn301-7) was mutagenized. Three separate populations of 10,000 seeds of transformant line Tn307-1 were treated with 0.3% EMS for 8–9 hr and were designated EMS1, EMS2, and EMS3. A population of 10,000 seeds of *lhy-1* that was not carrying the Tn301-7 transgene was mutagenized with EMS and designated EMS4. M1 plants were grown in pools of 25–50 plants. Approximately 20 M2 seeds per M1 plant were screened on GM agar for seedlings showing a wild-type hypocotyl. Those plants that flowered at a similar time to wild-type were selected for further study. The progeny of these plants were confirmed to be hygromycin resistant (carried by the *Ds* element in the *lhy-1* allele) and kanamycin resistant (carried by the T-DNA). To finally confirm that the *Ds* insertion characteristic of the *lhy-1* mutant allele was present in these plants, PCR was performed. Three such families derived from different mutagenesis populations were selected for detailed study. These were EMS1-3C, EMS2-23A, and EMS4-64A. M4 families of EMS1-3C and EMS2-23A were backcrossed to wild-type to remove the T-DNA containing the wild-type copy of *LHY*.

lhy-11 cca1-1, *lhy-12 cca1-1*, and *lhy-13 cca1-1* double null mutants were obtained by crossing and selecting for appropriate resistance markers (hygromycin resistance for *lhy* and kanamycin resistance for *cca1-1*). Prior to further analysis the double mutants were backcrossed to L*er* as follows. For RNA extraction for Northern blots, *lhy-12 cca1-1* and *lhy-11 cca1-1* were backcrossed three and four times, respectively, to L*er*. For flowering time and phenotypic analysis, *lhy-11 cca1-1*, *lhy-12 cca1-1*, and *lhy-13 cca1-1* were backcrossed four, five, and six times, respectively.

RNA Analysis and Analysis of Period Length

RNA (10 μg) was separated on 1.2% agarose denaturing formaldehyde gels and transferred to Hybond NX nylon membranes. Hybridization was done in 0.3 M sodium phosphate buffer (pH 7.0), 7% SDS, 1 mM EDTA, and 1% bovine serum albumin overnight at 65°C. The blot was washed twice for 20 min at 65°C with 0.2× SSC and 0.1% SDS. Probes were full-length *CCR2*, *LHY*, *CCA1*, *GI*, and *TOC1* cDNAs. The *COL1*- and *At5g17300*-specific probes were provided by Paul Reeves (John Innes Centre). Images were visualized using a Phosphorimager, and intensities were quantified using ImageQuant software. Values were represented relative to the lowest value of the wild-type samples after normalization to the 18S RNA control. All Northern blots were performed at least twice and usually with independent RNA samples. Period length was calculated as the time between peaks in mRNA abundance in adjacent cycles. For each genotype this calculation was performed in each of three cycles in two independent experiments, and the mean of these measurements was used to derive the period length as described (Green and Tobin, 1999).

Leaf Movement Analysis

Plants were sown on MS-agar plates and grown under light/dark cycles (12L 12D) for 10 days. Time-lapse video imaging was carried out in constant light, and the vertical position of the primary leaves was determined using the Kujata software (Millar et al., 1995b; Schaffer et al., 1998). Period and amplitude error calculations were performed by the fast Fourier transform nonlinear least square (FFT-NLLS) method (Plautz et al., 1997). More than one period may have been extracted from each data set, in which case only the most rhythmic was considered. Relative amplitude errors closer to 1 are indicative of weaker rhythmic signals.

Acknowledgments

We are grateful to R. Green and E. Tobin (UCLA) for providing the *cca1-1* mutant after backcrossing to L*er* and to Paul Reeves for providing the Atg17300 and COL1 probes. T.M. was funded by a long-term fellowship from the HFSPO. K.W. and L.W. received BBSRC and Gatsby Foundation studentships, respectively. Y.H. received an MPG Stipendium. Work in the laboratory of I.A.C. was supported by the BBSRC. The analysis of the *lhy* alleles was supported by a grant from the EC to G.C. within the REGIA program.

Received: November 26, 2001
Revised: March 14, 2002

References

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Más, P., and Kay, S.A. (2001). Reciprocal regulation between *TOC1* and *LHY/CCA1* within the *Arabidopsis* circadian clock. Science *293*, 880–883.

Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature *408*, 796–815.

Carpenter, C.D., Kreps, J.A., and Simon, A.E. (1994). Genes encoding glycine-rich Arabidopsis thaliana proteins with RNA-binding motifs are influenced by cold treatment and an endogenous circadian rhythm. Plant Physiol. *104*, 1015–1025.

Covington, M.F., Panda, S., Liu, X.L., Strayer, C.A., Wagner, D.R., and Kay, S.A. (2001). Elf3 modulates resetting of the circadian clock in arabidopsis. Plant Cell *13*, 1305–1316.

Devlin, P.F., and Kay, S.A. (2000). Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. Plant Cell *12*, 2499–2510.

Dunlap, J. (1999). Molecular bases for circadian clocks. Cell *96*, 271–290.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G., and Putterill, J. (1999). GIGANTEA: a circadian

clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J. *18*, 4679–4688.

Green, R.M., and Tobin, E.M. (1999). Loss of the circadian clock-associated protein 1 in Arabidopsis results in altered clock-regulated gene expression. Proc. Natl. Acad. Sci. USA *96*, 4176–4179.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science *290*, 2110–2113.

Heintzen, C., Nater, M., Apel, K., and Staiger, D. (1997). AtGRP7, a nuclear RNA-binding protein as a component of a circadian-regulated negative feedback loop in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA *94*, 8515–8520.

Hicks, K.A., Millar, A.J., Carre, I.A., Somers, D.E., Straume, M., Meeks-Wagner, D.R., and Kay, S.A. (1996). Conditional circadian dysfunction of the Arabidopsis early-flowering 3 mutant. Science *274*, 790–792.

Hicks, K.A., Albertson, T.M., and Wagner, D.R. (2001). Early flowering3 encodes a novel protein that regulates circadian clock function and flowering in Arabidopsis. Plant Cell *13*, 1281–1292.

Huq, E., Tepperman, J.M., and Quail, P.H. (2000). GIGANTEA is a nuclear protein involved in phytochrome signaling in Arabidopsis. Proc. Natl. Acad. Sci. USA *97*, 9654–9658.

Jarillo, J.A., Capel, J., Tang, R.H., Yang, H.Q., Alonso, J.M., Ecker, J.R., and Cashmore, A.R. (2001). An Arabidopsis circadian clock component interacts with both CRY1 and phyB. Nature *410*, 487–490.

Kreps, J.A., and Simon, A.E. (1997). Environmental and genetic effects on circadian clock-regulated gene expression in Arabidopsis. Plant Cell *9*, 297–304.

Ledger, S., Strayer, C., Ashton, F., Kay, S.A., and Putterill, J. (2001). Analysis of the function of two circadian-regulated CONSTANS-LIKE genes. Plant J. *26*, 15–22.

Liu, X.L., Covington, M.F., Fankhauser, C., Chory, J., and Wagner, D.R. (2001). ELF3 encodes a circadian clock-regulated nuclear protein that functions in an Arabidopsis PHYB signal transduction pathway. Plant Cell *13*, 1293–1304.

Makino, S., Kiba, T., Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Ueguchi, C., Sugiyama, T., and Mizuno, T. (2000). Genes encoding pseudo-response regulators: insight into His-to-Asp phosphorelay and circadian rhythm in Arabidopsis thaliana. Plant Cell Physiol. *41*, 791–803.

Makino, S., Matsushika, A., Kojima, M., Oda, Y., and Mizuno, T. (2001). Light response of the circadian waves of the APRR1/TOC1 quintet: when does the quintet start singing rhythmically in Arabidopsis? Plant Cell Physiol. *42*, 334–339.

Martinez-Garcia, J.F., Huq, E., and Quail, P.H. (2000). Direct targeting of light signals to a promoter element-bound transcription factor. Science *288*, 859–863.

McClung, C.R. (2001). Circadian rhythms in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. *52*, 139–162.

McWatters, H.G., Bastow, R.M., Hall, A., and Millar, A.J. (2000). The ELF3 zeitnehmer regulates light signalling to the circadian clock. Nature *408*, 716–720.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N.H., and Kay, S.A. (1995a). Circadian clock mutants in Arabidopsis identified by luciferase imaging. Science *267*, 1161–1163.

Millar, A.J., Straume, M., Chory, J., Chua, N.H., and Kay, S.A. (1995b). The regulation of circadian period by phototransduction pathways in Arabidopsis. Science *267*, 1163–1166.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., and Bartel, B. (2000). FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell *101*, 331–340.

Ouyang, Y., Andersson, C.R., Kondo, T., Golden, S.S., and Johnson, C.H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. Proc. Natl. Acad. Sci. USA *95*, 8660–8664.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A., and Nam, H.G. (1999). Control of circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. Science *285*, 1579–1582.

Plautz, J.D., Straume, M., Stanewsky, R., Jamison, C.F., Brandes, C., Dowse, H.B., Hall, J.C., and Kay, S.A. (1997). Quantitative analysis of Drosophila period gene transcription in living animals. J. Biol. Rhythms *12*, 204–217.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. (1995). The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell *80*, 847–857.

Samach, A., and Coupland, G. (2000). Time measurement and the control of flowering in plants. Bioessays *22*, 38–47.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carre, I.A., and Coupland, G. (1998). The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell *93*, 1219–1229.

Schaffer, R., Landgraf, J., Accerbi, M., Simon, V., Larson, M., and Wisman, E. (2001). Microarray analysis of diurnal and circadian-regulated genes in Arabidopsis. Plant Cell *13*, 113–123.

Somers, D.E., Devlin, P.F., and Kay, S.A. (1998a). Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. Science *282*, 1488–1490.

Somers, D.E., Webb, A.A., Pearson, M., and Kay, S.A. (1998b). The short-period mutant, toc1–1, alters circadian clock regulation of multiple outputs throughout development in Arabidopsis thaliana. Development *125*, 485–494.

Somers, D.E., Schultz, T.F., Milnamow, M., and Kay, S.A. (2000). ZEITLUPE encodes a novel clock-associated PAS protein from Arabidopsis. Cell *101*, 319–329.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A., and Kay, S.A. (2000). Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science *289*, 768–771.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature *410*, 1116–1120.

Wager-Smith, K., and Kay, S.A. (2000). Circadian rhythm genetics: from flies to mice to humans. Nat. Genet. *26*, 23–27.

Wang, Z.Y., and Tobin, E.M. (1998). Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. Cell *93*, 1207–1217.

Wang, Z.Y., Kenigsbuch, D., Sun, L., Harel, E., Ong, M.S., and Tobin, E.M. (1997). A Myb-related transcription factor is involved in the phtyochrome regulation of an *Arabidopsis* Lhcb gene. Plant Cell *9*, 491–507.

Yanovsky, M.J., Mazzella, M.A., and Casal, J.J. (2000). A quadruple photoreceptor mutant still keeps track of time. Curr. Biol. *10*, 1013–1015.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Valshnav, S., Li, Q., Sheng Sun, Z., Eichele, G., Bradley, A., and Lee, C.C. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell *105*, 683–694.