

Circadian Control of *cab* Gene Transcription and mRNA Accumulation in *Arabidopsis*

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An intriguing property of many organisms is their ability to exhibit rhythmic cellular events that continue independently of environmental stimuli. These rhythmic processes are generated by an endogenous mechanism known as the biological clock. We wished to determine whether *Arabidopsis thaliana* will serve as a model plant system for a molecular genetic dissection of the circadian clock. To this end, we investigated the expression of *Arabidopsis* chlorophyll *a/b*-binding protein (*cab*) genes throughout the circadian cycle. Steady-state mRNA levels of the *cab2* and *cab3* genes showed a dramatic circadian cycling in plants shifted from light/dark cycles to constant darkness, whereas the *cab1* mRNA level exhibited little or no cycling under the same conditions. Analysis of *cab* promoter fusions in transgenic tobacco revealed that both the *cab1* and *cab2* 5' upstream regions confer circadian-regulated expression on a chloramphenicol acetyltransferase (*cat*) reporter gene. In vitro nuclear run-on transcription assays also indicated that the transcription of the *cab1* and *cab2* genes is circadian regulated in *Arabidopsis*. Taken together, these data suggest that a post-transcriptional mechanism influences *cab1* mRNA levels in *Arabidopsis*. The identification of circadian-regulated *cis*-acting elements in the *cab1* and *cab2* upstream regions will provide powerful tools for both molecular and genetic analysis of the higher plant circadian clock.

INTRODUCTION

Many important cellular processes are observed to occur rhythmically with peak activity appearing once every 24 hr. These rhythms have been demonstrated to be under endogenous control, as they also persist under constant environmental conditions (Edmunds, 1988). The cellular machinery that generates circadian rhythms is known as the biological clock. Circadian rhythms have been observed in almost all eukaryotes appropriately examined (Rosbash and Hall, 1989) as well as in some prokaryotes (Huang et al., 1990). Interestingly, several properties of these rhythms are shared among these diverse species, including entrainment to natural day/night cycles by light and the ability to compensate for changes in ambient temperatures (Edmunds, 1988). An understanding of the molecular components that constitute these ubiquitous pacemakers will have, therefore, broad significance for many organisms. This is most pertinent where studies of circadian processes can be performed in organisms that are easily amenable to both genetic and molecular analysis, as has been shown in *Drosophila* (Rosbash and Hall, 1989; Young et al., 1989) and *Neurospora* (Dunlap, 1990).

Higher plants have played an important historical role in the study of biological clocks (Sweeney, 1987). Initially, the rhythmic "sleep" movements observed in leaves of

some legumes provided an excellent system for study (Simon et al., 1976; Millet et al., 1989). In addition to the morphological rhythms, plants have revealed numerous circadian oscillations at the physiological and biochemical levels. Processes as diverse as stem elongation (Lechamy and Wagner, 1984; Lechamy et al., 1990), stomatal opening and closure (Gorton et al., 1989), and many specific enzyme activities (Deng et al., 1989) exhibit circadian control. In most instances, these rhythms have been shown to be reset by light, and in several cases the photoreceptor that mediates the resetting stimulus has been shown to be phytochrome (Simon et al., 1976). However, the interaction between phytochrome and the circadian clock currently remains unclear.

More recently, the levels of specific mRNAs have been shown to be under circadian control in animal (Uhl and Reppert, 1986), fungal (Loros and Dunlap, 1991), and plant systems (Kay and Millar, 1990). In the case of higher plants, several investigators have documented the diurnal and circadian fluctuation in the level of mRNAs encoding the light-harvesting chlorophyll *a/b*-binding proteins of photosystem II (reviewed in Kay and Millar, 1990). Although these observations have been made in diverse species under different experimental regimes, several major properties can be summarized concerning circadian-regulated *cab* gene expression: *cab* mRNA levels peak in the late

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morning and reach a minimum in the late evening; this rhythmic fluctuation persists in constant darkness with a periodicity of approximately 24 hr but undergoes a significant damping in peak level; dramatic alteration of the light/dark regime can alter the phase of the cycling (Giuliano et al., 1988), and the cycling of *cab* mRNAs is more dramatic in some species than others (Meyer et al., 1989). The circadian clock has been shown to regulate *cab* gene expression at the transcriptional level, initially by study of the wheat *cab1* gene in transgenic tobacco (Nagy et al., 1988a) and later by in vitro nuclear run-on assays in several plant species (Giuliano et al., 1988; Paulsen and Bogorad, 1988; Taylor, 1989). These studies demonstrated that the clock acted directly upon transcription, and this also has been shown recently for the *Neurospora ccg* genes (Loros and Dunlap, 1991). In the case of the wheat *cab1* gene, a 268-bp promoter fragment has been shown to mediate clock-regulated, as well as phytochrome-regulated, transcription in transgenic tobacco (Nagy et al., 1988a; Fejes et al., 1990).

Although the identification of a *cis*-acting DNA element for circadian regulation is a major step forward in the molecular analysis of the biological clock, several problems hinder rapid progress in the plant field. First, with a few exceptions (Nagy et al., 1988a; Stayton et al., 1989), most investigators have analyzed the sum of the *cab* gene family transcription and, therefore, may have missed the possibility of differential regulation among individual family members. Second, the studies have not been performed in a plant species that can also offer a straightforward genetic approach to understanding the components of the clock. Therefore, we have initiated studies on circadian-regulated gene expression in *Arabidopsis* to determine whether this organism can be used as a model system for a molecular genetic analysis of the higher plant circadian clock. Besides its well-documented advantages for genetic analysis (Redei, 1970; Estelle and Somerville, 1986), *Arabidopsis* has a small and well-characterized *cab* gene family consisting of three highly homologous members (Leutweiler et al., 1986; Karlin-Neumann et al., 1988; Mitra et al., 1989) and a more divergent homolog that has been detected in DNA gel blots (Leutweiler et al., 1986).

The previous characterization of this gene family has allowed us to study the effect of the clock on specific *cab* genes. Surprisingly, one of these genes, *cab1*, did not exhibit an mRNA fluctuation, whereas the other two, *cab2* and *cab3*, showed a dramatic circadian cycling. Analysis of translational and transcriptional fusions of the *cab* promoters with a chloramphenicol acetyltransferase (*cat*) reporter gene in transgenic tobacco demonstrated that all three promoters are similarly regulated by the circadian clock. Consistent with these data, we also showed by using in vitro nuclear run-on transcription assays that the transcription rate of both the *cab1* and *cab2* genes is circadian regulated in *Arabidopsis*. We hypothesize, therefore, that post-transcriptional events result in a stabilization

of the *cab1* mRNA in *Arabidopsis* and conclude that this organism will provide a powerful system for both genetic and molecular dissection of the biological clock.

RESULTS

The Level of mRNA Encoding the *Arabidopsis cab* Gene Family Exhibits a Low Amplitude Circadian Cycling

It has been shown previously that some plant species exhibit a very poor cycling of *cab* mRNA throughout the circadian cycle (Meyer et al., 1989), preventing a study of the clock with this marker. Therefore, we investigated the cycling of *cab* mRNA in *Arabidopsis* to test whether the *cab* genes would be useful markers for studying circadian regulation in this species. Plants were grown from seed for 3 weeks in a growth chamber under a 12-hr light/12-hr dark (12L12D) regime. We began harvesting plants at the onset of illumination, and after 12 hr of illumination, the plants were placed in constant darkness (DD) for the following 50 hr. Samples were taken every 2 hr and frozen, and total RNA was prepared from each sample and analyzed for *cab* gene family mRNA in slot blots using a coding sequence probe. Figure 1 shows the results of this analysis. The sampling time was expressed in hours as zeitgeber time (ZT) (Zerr et al., 1990), which is simply the number of hours after the onset of illumination. Zeitgeber

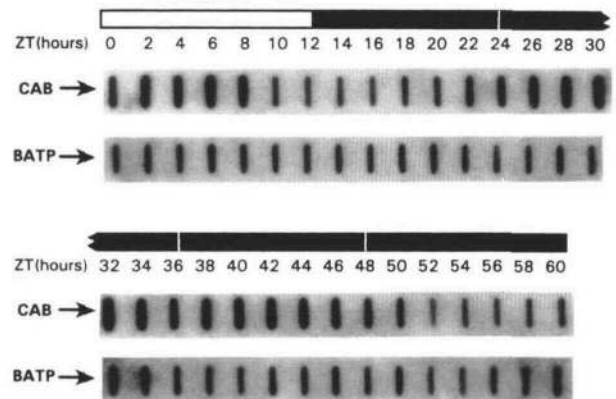


Figure 1. RNA Slot Blot Analysis of the *cab* Gene Family in *Arabidopsis*.

Total RNA was prepared from plants harvested every 2 hr during one LD cycle followed by DD. A slot blot of these samples was hybridized to coding region probes from *Arabidopsis cab1* (CAB) and the β -subunit of the mitochondrial ATPase (BATP). The open box above the time points marks the light period; the filled box indicates the period of constant darkness. Ten micrograms of RNA was loaded in each slot. ZT (hours), zeitgeber time (in hours).

is a common term used in chronobiology for the environmental signal(s) that resets the circadian clock. ZT is used to allow comparison of experiments conducted under different light/dark (LD) regimes (Zerr et al., 1990).

The *cab* mRNA level clearly fluctuated throughout the first cycle (ZT0 to ZT24), with a peak at ZT6 in the first day and another broad peak at ZT30 in DD (Figure 1). This periodicity of approximately 24 hr was similar to that observed for *cab* mRNA levels in several other plant species (Kay and Millar, 1990). In contrast to the *cab* mRNA level, no significant cycling in the level of the mRNA encoding the β -subunit of the mitochondrial ATPase could be detected (Figure 1). The amplitude of the *cab* mRNA level from peak (ZT6) to trough (ZT14) as determined by densitometry was only threefold, which is significantly lower than the 20-fold to 50-fold amplitude observed in other species such as wheat, tobacco, and tomato (Giuliano et al., 1988; Nagy et al., 1988a; Paulsen and Bogorad, 1988). These data demonstrated that the *Arabidopsis cab* gene family mRNA level is regulated by an endogenous circadian clock, albeit at a lower amplitude than some other species. The peak in *cab* mRNA level in DD was very broad, and only a shallow dip in expression level could be detected from ZT36 to ZT40. From ZT46 onward, the *cab* gene family mRNA level decreased to a basal level of 45% of the maximal, indicating a rapid damping of the *cab* mRNA rhythm in DD. In several other species, at least two peaks in *cab* mRNA level have been observed in DD (Kay and Millar, 1990).

Circadian Regulation of *Arabidopsis cab* mRNA Levels Is Gene Specific

From these results, it would appear that the *cab* mRNA level only cycles weakly in *Arabidopsis* and is not, therefore, a suitable marker for studying the circadian clock in this plant. However, the *Arabidopsis cab* gene family is composed of three major members: *cab1*, *cab2*, and *cab3* (Leutweiler et al., 1986; Mitra et al., 1989). All three genes are linked tightly, with *cab1* being transcribed in the opposite direction to the tandemly arranged *cab2* and *cab3* (Leutweiler et al., 1986). The coding regions of these three genes are conserved very highly at the nucleic acid level (96%), and the high homology between *cab2* and *cab3* extends into the 3' and 5' untranslated regions, as well as to approximately 190 bp upstream of the transcription start site (Leutweiler et al., 1986; Mitra et al., 1989). *cab1* has diverged from *cab2/cab3* in the noncoding regions of the gene. Because *cab2* and *cab3* are so highly homologous, it is technically difficult to distinguish between their respective mRNAs on a routine basis. Therefore, we prepared a single-stranded DNA probe that can distinguish between *cab1* and the *cab2/cab3* mRNAs in S1 nuclease protection experiments. This enabled us to investigate the

circadian regulation of the *cab* mRNAs on a gene-specific basis.

Figure 2 shows the results of an S1 nuclease protection analysis using the same RNA samples described in Figure 1. The signal derived from protection of the probe by *cab2/cab3* mRNAs shows a dramatic oscillation throughout the experimental period. Starting from ZT0, the *cab2/cab3* mRNA level peaked around ZT4 and declined to very low levels at ZT14, and a strong peak returned in DD around ZT28, indicating a periodicity of about 24 hr. An additional band is present between the *cab2/3* and *cab1* signals that is a breakdown product of the *cab2/3* band. The *cab2/cab3* mRNA levels varied by approximately 25-fold between maximum and minimum, and after ZT36 the signal declined to a very low level at ZT52. In striking contrast to the *cab2/cab3* mRNAs, the *cab1* mRNA remained relatively constant throughout the entire experimental period, with no more than a twofold variation and a small decline observed at ZT54.

Taken together, these results demonstrated that the *cab2* and *cab3* mRNA levels are strongly regulated in a circadian fashion, whereas the *cab1* mRNA level is only weakly, if at all, regulated by the circadian clock. The summation of these signals at each time point resulted in the low amplitude fluctuation observed in the slot blot analysis shown in Figure 1. Although minor differences in the cycling of specific *cab* mRNAs had been reported previously in petunia (Stayton et al., 1989), all of the steady-state *cab* mRNA levels exhibited clear cycling, unlike our observations for the *cab1* gene in *Arabidopsis*.

Transcription of Both the *cab1* and *cab2* Genes Is Regulated by the Circadian Clock

The differential expression of the *Arabidopsis cab* genes during the circadian cycle could result from differences in either transcription, mRNA turnover, or both during the circadian cycle. To investigate which of these possibilities were operating, we examined the expression of both translational and transcriptional *cab-cat* fusions in transgenic tobacco. Seeds of the translational fusions were kindly provided by Dr. Gynheung An (Washington State University), who has demonstrated that these fusions are correctly expressed in transgenic tobacco in terms of light/dark and organ-specific regulation (An, 1987). The transgenic tobacco seeds were germinated and grown for 2 weeks under a 16L8D regime, after which the seedlings were entrained to 12L12D for 48 hr. Samples of seedlings were collected and frozen every 3 hr from an initial LD cycle, followed by continuous darkness. RNA was isolated and the expression of the chimeric *cab-cat* mRNA was assayed in RNA gel blots using a radiolabeled probe derived from the *cat* gene.

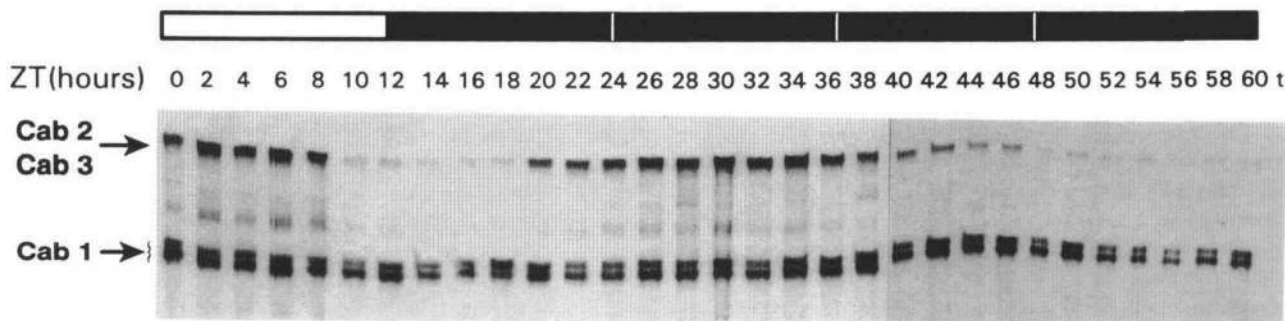


Figure 2. S1 Nuclease Protection Analysis of the *cab* Family Members in *Arabidopsis*.

The RNA samples described in Figure 1 were hybridized to a probe from the 5' end of the *cab2* gene. The *cab2* and *cab3* protection products migrate at the same position (Cab2/Cab3); the *cab1* protection products migrate as a double band (Cab1). Open box, light period; filled box, dark period. Each sample contained 25 μ g of total RNA.

Figure 3 shows the results of a typical experiment from plants bearing either a *cab1-cat* or *cab2-cat* translational fusion. The *cab1-cat* construct exhibited a weak oscillation in the LD cycle, with only a sevenfold difference between maximum (ZT0 and ZT27) and the minimum (ZT15) expression levels. In DD, however, we observed a broad plateau in *cat* mRNA which remained high at least until ZT42. As a control, the level of mRNA encoding the β -subunit of the endogenous mitochondrial ATPase was assayed on the same blot and was constant throughout the entire experimental period.

In contrast to the *cab1-cat* expression pattern, the *cab2-cat* translational construct was clearly under circadian regulation, as shown in Figure 3B. The mRNA derived from this construct peaked at ZT3 and at ZT24 and had well-defined peaks in both LD and DD. Expression of this construct declined to a low level after ZT33, and no significant change was observed in the level of the control β -subunit mRNA throughout this period. All of the independent transformants we assayed gave an expression pattern identical to that shown in Figures 3A and 3B (data not shown). We also analyzed similar *cab3-cat* translational fusions that showed expression patterns identical to those of the *cab2-cat* constructs shown in Figure 3B (data not shown). These data demonstrated that sequences residing between -734 and $+462$ of the *Arabidopsis cab2* gene are sufficient to confer circadian regulation on the *cat* reporter gene in transgenic tobacco. In the case of the *cab1* construct (Figure 3A), only a weak diurnal regulation could be observed, with no detectable cycling in DD.

To investigate further the differential regulation of the *cab1* and *cab2* genes, we made constructs consisting of transcriptional fusions between the respective *cab* 5' upstream regions and the *cat* reporter gene. No *Arabidopsis cab* mRNA coding sequence is present in these constructs except for 6 bases of *cab1* and 3 bases of *cab2* from the

respective 5' leaders; this allowed us to test whether the clock is acting at the transcriptional level. These constructs were transferred to tobacco, and young R0 plants from several independent transformants were analyzed for circadian activity of the *cab* promoters, as described above for the translational fusions. Figure 4A shows the results of such an analysis for plants containing a construct extending from -249 to $+6$ of the *cab1* promoter fused to *cat* and sampled at 4-hr intervals. In sharp contrast to the translational *cab1* fusion (Figure 3A), the transcriptional fusion showed a clear circadian fluctuation in both LD and DD (Figure 4A). In LD, the first peak in *cat* mRNA occurred at ZT4 and the second peak in the ZT28 sample in DD, whereas the mRNA level declined to a low level at ZT32. No broad plateau in mRNA level occurred in DD as we observed for the translational fusion (Figure 3A). The amplitude of expression between the maxima (ZT4 and ZT28) and minima (ZT12 and ZT32) was 25-fold, which is much larger than that observed for the translational fusion. Some damping of the rhythm is evident because the peak at ZT28 was only 70% of the peak at ZT4, as would be expected for circadian regulation. As a control, we analyzed the expression of a cauliflower mosaic virus 35S promoter- β -glucuronidase (*gus*) reference gene that is also present on the T-DNA, and we found that it does not exhibit circadian-regulated expression (Figure 4A).

We also analyzed the expression of a construct containing the *cab2* 5' upstream region extending from -319 to $+3$ fused to the *cat* coding sequence in the same vector used for the *cab1-cat* transcriptional fusion described above. Transgenic tobacco seedlings from several independent transformants bearing this construct were analyzed by RNA gel blot analysis, and Figure 4B shows a typical example. The expression of this transcriptional *cab2-cat* chimeric gene was regulated in a circadian fashion in a pattern very similar to that observed for the

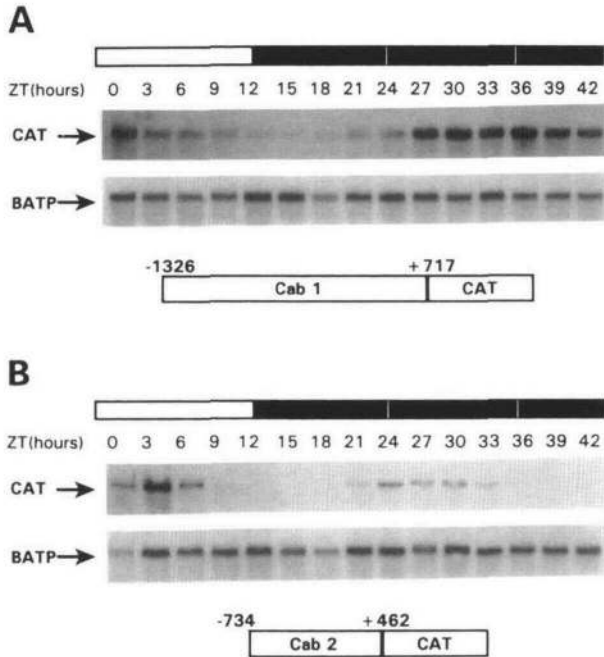


Figure 3. RNA Gel Blot Analysis of *cab-cat* Translational Fusions in Transgenic Tobacco.

(A) *cab1-cat* translational fusion.

(B) *cab2-cat* translational fusion.

Total RNA was prepared from the relevant transgenic plants harvested at the times indicated. An RNA gel blot of these samples was hybridized to coding region probes from *cat* (CAT) and the β -subunit of the mitochondrial ATPase (BATP). Open box, light period; filled box, dark period. Boxes below each part: nucleotide positions of *cab* fragments used in each construction, and relative length of the *cat* coding region. Twenty micrograms of total RNA was loaded in each lane.

translational *cab2-cat* fusion described earlier (Figure 3B) and the transcriptional *cab1-cat* fusion (Figure 4A). The first peak in the *cat* mRNA level occurred around ZT3 in LD and the second peak around ZT27 in DD, with the second peak damped by 35% relative to the first. The peak mRNA level at ZT3 was 50-fold greater than the trough at ZT15.

To confirm that the transcription rate of the *cab1* and *cab2* genes is circadian regulated in *Arabidopsis*, we performed *in vitro* nuclear run-on transcription assays. Nuclei were prepared from *Arabidopsis* plants through one cycle of LD, followed by one cycle of DD. The nuclei were used to generate nascent radiolabeled transcripts *in vitro*, which were then hybridized to filters bearing single-strand DNA probes derived from either the *cab1* or *cab2* genes, or rDNA as a control. Figure 5 shows the results of a typical experiment, where transcription rate is represented as the fraction of total input 32 P-RNA that hybridized to each

probe. Both the *cab1* and *cab2* genes exhibited a transcription rate that dropped markedly from ZT4 to a trough at ZT10 to ZT16 and peaked again at ZT28 in DD, indicating circadian regulation (Figure 5A). We note that the timing of peaks and troughs appears to precede by several hours the corresponding maxima and minima in mRNA levels of the *cab2* gene assayed under a similar regime (Figure 2). However, the sampling interval of the nuclei is 6 hr, and more frequent time points may be necessary to resolve more accurately the circadian fluctuations in transcription rate. Because of the different probes used for the *cab1* and *cab2* genes and a low level of cross-hybridization (see Methods), no conclusions can be drawn regarding the relative magnitude of transcription rate between these two genes. In marked contrast to the *cab* genes, the transcription rate of the rDNA genes appears to exhibit an erratic fluctuation of no more than 1.5-fold throughout the experimental period (Figure 5B). This is very consistent with fluctuations observed for rDNA transcription rate in nuclei isolated from oat seedlings (Lissemore and Quail, 1988).

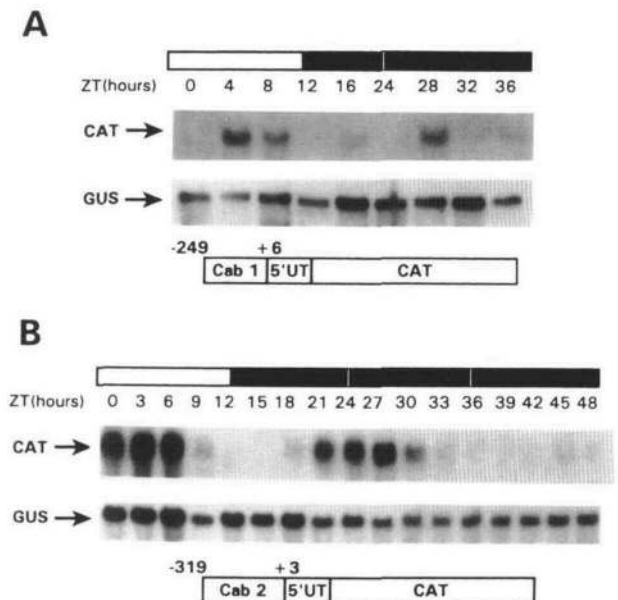


Figure 4. RNA Gel Blot Analysis of *cab-cat* Transcriptional Fusions in Transgenic Tobacco.

(A) *cab1-cat* transcriptional fusion.

(B) *cab2-cat* transcriptional fusion.

Total RNA prepared from plants harvested at the times indicated was hybridized to coding region probes from *cat* (CAT) and *gus* (GUS). Open box, light period; filled box, dark period. Boxes below each part: nucleotide positions of *cab* fragments used in each construction, and relative length of the *cat* coding region. 5'UT, 5' untranslated leader. Twenty micrograms of total RNA was loaded in each lane.

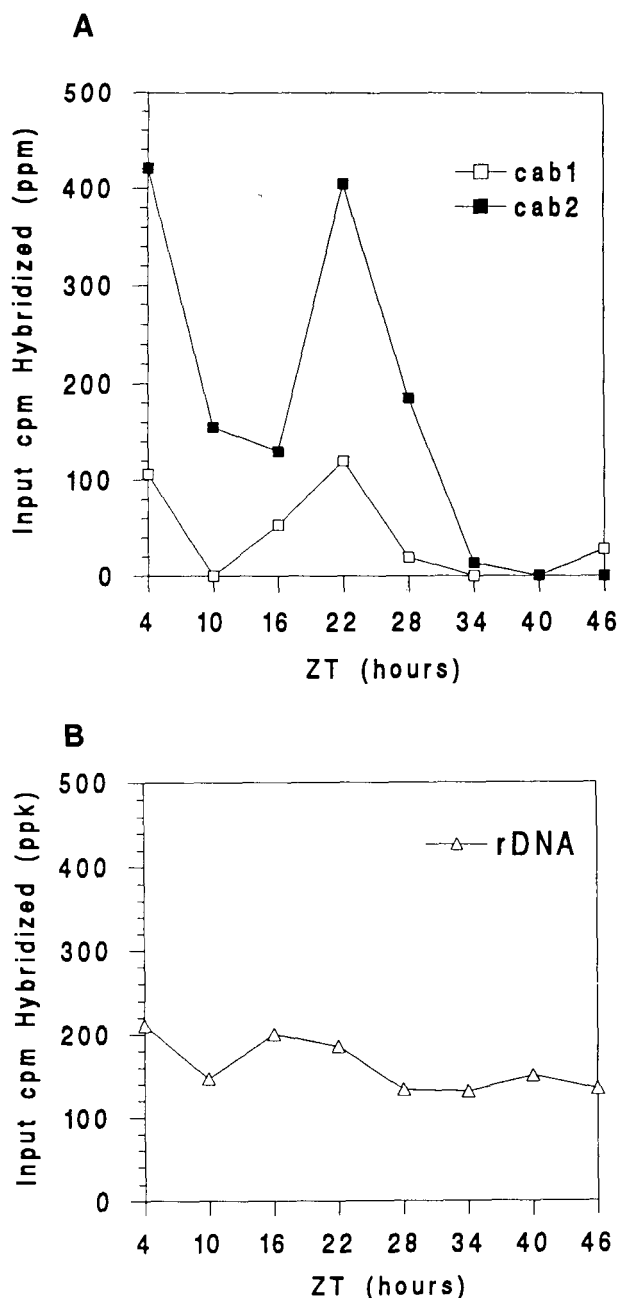


Figure 5. In Vitro Nuclear Run-On Assays of *cab* Gene Transcription in *Arabidopsis*.

(A) *cab1* and *cab2* transcription assays.

(B) rDNA transcription assays.

In vitro transcripts from nuclei prepared at the times indicated were hybridized to probes derived from the *cab1* and *cab2* 3' untranslated regions (□, ■) and to rDNA (△). Lights were on from ZT0 to ZT8 during plant harvesting, followed by constant darkness to ZT46. Data for rRNA hybridization is presented as parts per thousand (ppk).

Taken together, all of these data demonstrated that transcription of both the *Arabidopsis cab1* and *cab2* genes is regulated by the circadian clock and occurs in a similar phase. The transcriptional regulation is mediated by *cis*-acting elements residing between -249 and $+6$ in *cab1* and -319 and $+3$ in *cab2*. Furthermore, a post-transcriptional mechanism may be involved in determining steady-state *cab1* mRNA levels in *Arabidopsis* because the mRNA level does not oscillate significantly but remains relatively constant throughout the diurnal cycle.

DISCUSSION

The study of circadian clocks has been facilitated in recent years by the concerted application of both genetic and molecular techniques. Genetic loci that are required for normal clock functions have been identified and cloned in *Drosophila* (Rosbash and Hall, 1989; Young et al., 1989) and *Neurospora* (Dunlap, 1990). Such loci provide powerful tools for the study of the cellular components of biological clocks. In higher plants, genes required for clock function have not yet been identified, but the discovery of clock-regulated mRNAs and *cis*-acting elements (Nagy et al., 1988a) provides a molecular assay for the circadian clock. We have demonstrated here that clock function can be assayed using relatively small promoter fragments derived from members of the *Arabidopsis cab* gene family. This will allow the consolidation of molecular and genetic approaches to study clocks in a model system for higher plants. Interestingly, we have also demonstrated that the steady-state level of *cab1* mRNA does not cycle in *Arabidopsis*, whereas the *cab1* promoter confers circadian-regulated expression in transgenic tobacco. This result provides a clear example of the importance of post-transcriptional events in determining steady-state mRNA levels of specific plant genes, which is currently an area of intense interest (Walling et al., 1986; Dean et al., 1989; Ingelbrecht et al., 1989; Silverthorne and Tobin, 1990).

Our results showed that the *cab1* upstream region confers high amplitude cycling on the *cat* reporter gene in transcriptional fusions (Figure 4A), whereas translational *cab1-cat* fusions exhibit a low amplitude rhythm in LD (Figure 3A) and the endogenous steady-state *cab1* mRNA level exhibits a very weak oscillation, if any. Several factors must be considered that may account for these data. First, the upstream regions present in the translational and transcriptional fusions are different, and this may be the only reason for the observed differences in reporter gene expression. The *cab1-cat* translational fusion contains 1326 bp of 5' upstream region, whereas the *cab1-cat* transcriptional fusion only contains 249 bp upstream of the transcriptional initiation site (Figures 3A and 4A). Sequences present in the additional 1 kb of upstream region,

therefore, may have given rise to the relatively low amplitude cycling observed with the translational fused construct. We consider this explanation unlikely because we have also assayed the expression of a *cab1-cat* translational fusion (provided by Dr. An) containing only 251 bp of upstream sequence and this construct exhibited an expression pattern of low amplitude cycling identical to that observed for the -1326 fusion (A. J. Millar and S. A. Kay, unpublished results). Second, the vector used for the *cab-cat* translational constructs is different from that used for our transcriptional fusions, creating different 3' untranslated regions in the chimeric mRNAs that may have also influenced our results. However, the *cab2-cat* fusions gave an identical expression pattern regardless of vector background (Figures 3B and 4B), indicating that this did not have a significant effect on our data.

Another possible explanation for the discrepancy between the *cab1* promoter expression pattern and the steady-state *cab1* mRNA level in *Arabidopsis* is that the promoter constructs were all assayed in transgenic tobacco rather than in *Arabidopsis*. Thus, the *cab1* promoter could be regulated inappropriately in tobacco. To investigate this, we performed in vitro nuclear run-on experiments in *Arabidopsis* to test for *cab* transcriptional activity throughout a circadian cycle in DD and found that the transcription of both the *cab1* and *cab2* genes cycles throughout this period (Figure 5). Therefore, it is highly likely that the *cab1* promoter is circadian responsive in both *Arabidopsis* and transgenic tobacco. In further support of this conclusion, previous studies have shown that all three *Arabidopsis cab* gene promoters exhibit a pattern of expression in transgenic tobacco very similar to that observed in *Arabidopsis* (Ha and An, 1988; Mitra et al., 1989).

The most plausible hypothesis that accounts for our data is that sequences downstream of +6 in the *cab1* gene are required for a post-transcriptional mechanism that results in *cab1* mRNA levels remaining relatively constant throughout the diurnal cycle. This occurs despite the clock-regulated transcription of the *cab1* gene. Interestingly, the sequences mostly responsible for this effect may reside in the 5' untranslated leader of the *cab1* gene. Support for this notion comes from analysis of the translational *cab-cat* fusions. The transcribed *cab* coding sequences present in these constructs extend from the initiation codon at +71 to +717 in the case of *cab1* and from the initiation ATG at +54 to +462 for the *cab2* construct. The *cab1* and *cab2* coding sequences from the ATG to +717 are 96% homologous with only single-base pair mismatches (Leutweiler et al., 1986). Thus, the difference in expression pattern of *cab1-cat* versus *cab2-cat* translational fusions is unlikely to be due to the Cab protein coding sequences retained in these constructs.

A more plausible candidate responsible for the differential expression pattern is the 5' untranslated leader of *cab1*, which is divergent from the *cab2* and *cab3* leader

sequences. If this hypothesis were true, it would be one of the first examples in which the 5' untranslated mRNA leader has been implicated in regulating mRNA levels in higher plants. Future experiments placing the *cab1* leader in *cab2-cat* fusions will determine whether the *cab1* 5' leader has an independent role in post-transcriptional regulation. We note that the *cab1-cat* translational fusion exhibits a higher amplitude circadian expression pattern than the steady-state *cab1* mRNA level observed in *Arabidopsis*. This difference could be because of two hypothetical mechanisms. First, the translational *cab1-cat* construct (Figure 3A) may require additional sequences from the 3' portion of the *cab1* gene to generate the expression pattern observed in *Arabidopsis* (Figure 2). Second, the presence of the *cat* mRNA coding sequences, which we have shown previously to be relatively unstable in transgenic tobacco (Nagy et al., 1988a), may give rise to some of the cycling seen in the *cab1-cat* chimeric RNA. We cannot yet account for the observation that the translational *cab1-cat* fusion does not cycle at all in constant darkness (Figure 3A) but shows a low amplitude cycling in LD. Further experiments will determine the expression pattern of the chimeric *cab1-cat* mRNA in extended darkness.

The physiological consequences of circadian-regulated *cab* gene expression in *Arabidopsis* are currently unknown. We have proposed previously (Nagy et al., 1988a; Kay and Millar, 1990) that cycling of *cab* mRNA may be a mechanism by which synthesis of *cab* apoprotein could be coupled to light-regulated chlorophyll biosynthesis. This could be important in both the biogenesis and maintenance of light-harvesting complexes in vivo. However, it is unclear why the *cab1* gene is clock regulated at the transcriptional level when this control does not manifest itself at the level of steady-state *cab1* mRNA. Our data have demonstrated that the closely related *cab2* and *cab3* genes are cycling at the transcriptional level as well as at the level of mRNA. All three *cab* genes encode identical mature proteins, which we presume are functionally equivalent, but it is not known whether all three genes are expressed in the same cell types. Previous experiments showed that the *cab1* gene is induced by light to a greater extent than the *cab2/3* in etiolated tissue (Karlin-Neumann et al., 1988). However, it has not been demonstrated whether the differential regulation is exerted at the level of transcription or by post-transcriptional events. This expression pattern may have evolved to deliver the *cab1* and *cab2/3* gene products to distinct groups of cells at appropriate times. We are currently investigating the cell-specific expression pattern of the individual *Arabidopsis cab* genes using upstream regions fused to luciferase (Koncz et al., 1990) reporter genes.

The upstream *cis*-elements identified in the wheat *cab1* gene (Nagy et al., 1988a; Fejes et al., 1990) and the three *Arabidopsis cab* genes reported here are the only such elements that are known to confer circadian-regulated

transcription. *per* mRNA has been shown to cycle in *Drosophila* (Hardin et al., 1990), and clock-controlled genes (*ccgs*) that are transcriptionally regulated have been identified in *Neurospora* (Loros and Dunlap, 1991), but no upstream elements conferring this regulation have yet been identified. The *cab* circadian-responsive *cis*-elements that we have identified in *Arabidopsis* will allow two fundamental approaches for dissecting the circadian clock. The first is a molecular approach that will allow a more detailed definition of the clock-responsive elements, which in turn can be used to assay for putative protein factors whose activity is regulated in a time-dependent manner. Such proteins are already known to exist, as the level of a mammalian transcription factor, DBP, was shown to be circadian regulated in rat hepatocytes (Wuarin and Schibler, 1990). The second approach is a genetic analysis in which the *cab* upstream region, fused to either screenable markers [such as luciferases *lux* (Koncz et al., 1987) and *luc* (Schneider et al., 1990) or *gus* (Jefferson et al., 1987)], or selectable markers, such as hygromycin phosphotransferase, will allow identification of loci that are responsible for correct clock function in *Arabidopsis*.

METHODS

Plant Material

Arabidopsis thaliana plants (ecotype Columbia *gl1*, seed supplied by Guhy's Specialty Nursery, Tucson, AZ) were grown in a growth chamber at a constant 22°C under a 12-hr photoperiod (12L12D) for 3 weeks (RNA analysis) or under 8L16D for 5 weeks (nuclear isolation). R1 seeds of transformed tobacco (var Xanthi) carrying *cab-cat* translational fusions (An, 1987) were grown in sterile culture on MS medium (Murashige and Skoog, 1962) containing kanamycin at 23°C for 2 weeks under 16L8D, then for 2 days in 12L12D. Tobacco plants (var SR1) were maintained in sterile culture on MS medium to provide leaves for transformation experiments. Regenerated kanamycin-resistant plants carrying the transcriptional *cab-cat* fusions were propagated in sterile culture and entrained to 12L12D as for the R1 plants. Three to five independently transformed lines that expressed the transgene at high levels were assayed for each of the transcriptional and translational fusions.

RNA Extraction and Analysis

Total RNA was prepared from all aerial parts of the plants (*Arabidopsis* and translational *cab-cat* fusions) or from leaf tissue (transcriptional fusions) and analyzed in slot blots, RNA gels, or S1 nuclease protection assays, as described previously (Nagy et al., 1988b). The coding region probes used in the slot blots and RNA gel blots were from the β -subunit of the mitochondrial ATPase (Boutry and Chua, 1985) and a 537-bp *Avall* fragment from *cab1*. The probe used in Figure 2 was a 640-bp *ClaI*-*Bam*HI fragment from the 5' end of the *cab2* gene, uniformly labeled with ³⁵S-deoxyadenosine. Hybridization for the S1 nuclease protection

assays was performed with 25 μ g of total RNA in 80% formamide/0.4 M NaCl at 47°C, with digestion in 800 units/mL S1 nuclease for 90 min at room temperature. The double band produced by the *cab1* mRNA protection product (about 155 nucleotides) is a property specific to this probe; the *cab2* and *cab3* protection products (206 nucleotides) comigrate. Autoradiograms of the slot blots, RNA gel blots, and S1 analysis were quantitated by densitometry using an LKB Ultrascan densitometer. The amplitude of *cab* or *cat* mRNA cycling in the slot blots and RNA gel blots was calculated from the ratios of *cab* or *cat* to control (β -ATPase or *gus*) density for the relevant time points. The amplitude of mRNA cycling in the S1 analysis was calculated from the absolute density of the relevant bands.

Clones and Construction

Arabidopsis genomic clones containing the *cab1* (AB140) and *cab2* (AB165) genes were kindly provided by Dr. Elaine Tobin (University of California, Los Angeles). All nucleotide positions are calculated using the transcription start sites defined previously (Karlin-Neumann et al., 1988) as the +1 position. The R1 tobacco seeds kindly provided by Dr. Gynheung An carry *cat* translational fusions 568 (−1326 to +717 of *cab1*), 499 (−734 to +462 of *cab2*), and 500 (−900 to +462 of *cab3*). Briefly, the translational fusions consist of the relevant *cab-cat* fusion described above in the binary *cat* expression vector pGA534 and have been described in detail (An, 1987). All the translational fusions contain the same *cat* sequences and octopine T-DNA 3' termination signal. The transcriptional fusions to *cat* contain the following sequences: in *cab1-cat*, a *Bgl*II-*Sfa*NI fragment (−249 to −3) of *cab1* is fused to an oligonucleotide that reconstitutes the *cab1* sequence from −249 to +6, followed by a *Bsp*MI site, 29 bp of the wheat *cab1* 5' untranslated region, and a *Sal*I site at position +47 of the fusion construct. The heterologous 5' untranslated region was included to eliminate possible post-transcriptional effects of *Arabidopsis* mRNA sequences on mRNA abundance. Nagy et al. (1988a) demonstrated that the abundance of the wheat *cab1* transcript does not cycle in transgenic tobacco when fused to a constitutive promoter. The *cab2-cat* transcriptional fusion contains a *Taq*I fragment of *cab2* (−319 to −16) fused to an oligonucleotide that reconstitutes *cab2* sequence from −319 to +3, followed by sequences identical to the *cab1-cat* construct from +4 to the *Sal*I site. These promoter-leader constructs were ligated to an identical *cat* gene bearing the pea *rbcS-E9* transcriptional terminator in the pUC-based plasmid p827, provided by Dr. Pam Green (Michigan State University), and each *cab-cat-E9* fusion was transferred to the polylinker of the binary vector pMON721 (provided by Monsanto Corporation, St. Louis, MO). A reference gene consisting of the cauliflower mosaic virus 35S promoter from position −941, fused to the *gus* gene with the pea *rbcS-3C* transcriptional terminator, was introduced into the *Not*I site at position 4836 of pMON721, reading on the opposite strand from the *cab-cat* fusion.

In Vitro Nuclear Run-On Transcription Assays

Nuclei were isolated at 6-hr intervals from all aerial parts of prefloral *Arabidopsis* plants as described (Green et al., 1988). The time to prepare nuclei, from tissue homogenization to freezing,

was approximately 30 min. In vitro nuclear run-on assays were performed essentially as described (Lissemore and Quail, 1988). The *cab* 3' untranslated region probes were isolated by polymerase chain reaction from genomic DNA and several independent clones were sequenced for each construct. The *cab1* probe corresponds to nucleotides +767 to +866. The *cab2* probe spanned the 1.6-kb region between *cab2* and *cab3*, from position +769 of *cab2* to position -878 of *cab3*. The *Arabidopsis* rDNA clone pARR17 was kindly provided by Dr. Eric Richards (Cold Spring Harbor). Single-stranded DNA probes and ³⁵S in vitro transcripts were prepared from clones in pBluescript vectors, as recommended by the manufacturer (Stratagene). Single-stranded DNA probes were immobilized on a nitrocellulose filter using a slot-blot manifold. Filters were cut and individual slots hybridized in 1.5-mL microcentrifuge tubes. Hybridization and washing conditions were as described (Lissemore and Quail, 1988) except that the final wash was at 37°C in 1 × SSC/0.5% SDS. *cab* and rRNA hybridizations were normalized by subtracting ³²P hybridization to pBluescript as a background control. Controls were also performed using ³⁵S-RNAs to test for cross-hybridization between the single-stranded DNA probes and RNA from the noncognate *cab* gene. Hybridization of input probe counts per minute after background subtraction were 13% for the *cab2* RNA on the *cab1* probe and 28% for the *cab1* RNA on the *cab2* probe. Hybridization efficiency of *cab* and rDNA transcripts was calculated by incorporating a known amount of ³⁵S RNA in each of the hybridization reactions. The assays were repeated six times and gave consistent results. Figure 5 shows the results of typical experiments, in which hybridization efficiencies ranged from 26% to 40% for *cab* probes and 42% to 74% for the rDNA probes. The *cab* and rRNA data in this figure are derived from separate assays of the same nuclear samples.

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REFERENCES

- An, G. (1987). Integrated regulation of the photosynthetic gene family from *Arabidopsis thaliana* in transformed tobacco cells. *Mol. Gen. Genet.* **207**, 210–216.
- Boutry, M., and Chua, N.-H. (1985). A nuclear gene encoding the β subunit of the mitochondrial ATPase in *N. plumbaginifolia*. *EMBO J.* **4**, 2159–2165.
- Dean, C., Favreau, M., Bond-Nutter, D., Bedbrook, J., and Dunsmuir, P. (1989). Sequences downstream of translation start regulate quantitative expression of two petunia *rbcS* genes. *Plant Cell* **1**, 201–208.
- Deng, M.-D., Moureaux, T., and Lamaze, T. (1989). Diurnal and circadian fluctuation of malate levels and its close relationship to nitrate reductase activity in tobacco leaves. *Plant Sci.* **65**, 191–197.
- Dunlap, J.C. (1990). Closely watched clocks: Molecular analysis of circadian rhythms in *Neurospora* and *Drosophila*. *Trends Genet.* **6**, 159–165.
- Edmunds, L.N. (1988). Cellular and Molecular Bases of Biological Clocks. (New York: Springer-Verlag).
- Estelle, M.A., and Somerville, C. (1986). The mutants of *Arabidopsis*. *Trends Genet.* **2**, 89–93.
- Fejes, E., Pay, A., Kanevsky, I., Szell, M., Adam, E., Kay, S., and Nagy, F. (1990). A 268 bp upstream sequence mediates the circadian clock-regulated transcription of the wheat *cab-1* gene in transgenic plants. *Plant Mol. Biol.* **15**, 921–932.
- Giuliano, G., Hoffman, N.E., Ko, K., Scolnik, P.A., and Cashmore, A.R. (1988). A light-entrained circadian clock controls transcription of several plant genes. *EMBO J.* **7**, 3635–3642.
- Gorton, H.L., Williams, W.E., Binns, M.E., Gemmell, C.N., Leheny, E.A., and Shepherd, A.C. (1989). Circadian stomatal rhythms in epidermal peels from *Vicia faba*. *Plant Physiol.* **90**, 1329–1334.
- Green, P.J., Kay, S.A., Lam, E., and Chua, N.-H. (1988). In vitro DNA footprinting. In *Plant Molecular Biology Manual*, S. Gelvin and R. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–22.
- Ha, S.-B., and An, G. (1988). Identification of upstream regulatory elements involved in the developmental expression of the *Arabidopsis thaliana cab1* gene. *Proc. Natl. Acad. Sci. USA* **85**, 8017–8021.
- Hardin, P.E., Hall, J.C., and Rosbash, M. (1990). Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature* **343**, 536–540.
- Huang, T.C., Tu, J., Chow, T.-J., and Chen, T.-H. (1990). Circadian rhythm of the prokaryote *Synechococcus* sp. RF-1. *Plant Physiol.* **92**, 531–533.
- Ingelbrecht, I.L.W., Herman, L.M.F., Dekeyser, R.A., Van Montagu, M.C., and Depicker, A.G. (1989). Different 3' end regions strongly influence the level of gene expression in plant cells. *Plant Cell* **1**, 671–680.
- Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β -Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.
- Karlin-Neumann, G.A., Sun, L., and Tobin, E.M. (1988). Expression of light-harvesting chlorophyll *a/b*-protein genes is phytochrome-regulated in etiolated *Arabidopsis thaliana* seedlings. *Plant Physiol.* **88**, 1323–1331.
- Kay, S. A., and Millar, A. J. (1990). Circadian regulated *cab* gene transcription in higher plants. In *The Molecular Biology of Circadian Rhythms*, M. Young, ed (New York: Marcel Dekker), in press.

- Koncz, C., Olsson, O., Langridge, W.H.R., Schell, J., and Szalay, A.A.** (1987). Expression and assembly of functional bacterial luciferase in plants. *Proc. Natl. Acad. Sci. USA* **84**, 131–135.
- Koncz, C., Langridge, W.H.R., Olsson, O., Schell, J., and Szalay, A.A.** (1990). Bacterial and firefly luciferase genes in transgenic plants: Advantages and disadvantages of a reporter gene. *Dev. Genet.* **11**, 224–232.
- Lechary, A., and Wagner, E.** (1984). Stem elongation rate in light-grown plants: Evidence for an endogenous circadian rhythm in *Chenopodium rubrum*. *Physiol. Plant.* **60**, 437–453.
- Lechary, A., Tremolières, A., and Wagner, E.** (1990). Correlation between the endogenous circadian rhythmicity in growth rate and fluctuations in oleic acid content in expanding stems of *Chenopodium rubrum* L. *Planta* **182**, 211–215.
- Leutweiler, L.S., Meyerowitz, E.M., and Tobin, E.M.** (1986). Structure and expression of three light-harvesting chlorophyll *a/b*-binding protein genes in *Arabidopsis thaliana*. *Nucl. Acids Res.* **14**, 4051–4064.
- Lissemore, J.L., and Quail, P.H.** (1988). Rapid transcriptional regulation of the genes for phytochrome and chlorophyll *a/b*-binding protein in *Avena sativa*. *Mol. Cell. Biol.* **8**, 4840–4850.
- Loros, J.J., and Dunlap, J.C.** (1991). *Neurospora crassa* clock-controlled genes are regulated at the level of transcription. *Mol. Cell. Biol.* **11**, 558–563.
- Meyer, H., Thienel, U., and Piechulla, B.** (1989). Molecular characterization of the diurnal/circadian expression of the chlorophyll *a/b*-binding proteins in leaves of tomato and other dicotyledonous and monocotyledonous plant species. *Planta* **180**, 5–15.
- Millet, B., Coillot, L., and Agosti, R.D.** (1989). The rhythmic leaf movements after regeneration of partially excised pulvinus in *Phaseolus vulgaris* L. *Plant Cell Physiol.* **30**, 643–648.
- Mitra, A., Choi, H.K., and An, G.** (1989). Structural and functional analyses of *Arabidopsis thaliana* chlorophyll *a/b*-binding protein (*cab*) promoters. *Plant Mol. Biol.* **12**, 169–179.
- Murashige, T., and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15**, 493–497.
- Nagy, F., Kay, S.A., and Chua, N.-H.** (1988a). A circadian clock regulates transcription of the wheat *cab-1* gene. *Genes Dev.* **2**, 376–382.
- Nagy, F., Kay, S.A., and Chua, N.-H.** (1988b). Analysis of gene expression in transgenic plants. In *Plant Molecular Biology Manual*, S. Gelvin and R. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–29.
- Paulsen, H., and Bogorad, L.** (1988). Diurnal and circadian rhythms in the accumulation and synthesis of mRNA for the light-harvesting chlorophyll *a/b*-binding protein in tobacco. *Plant Physiol.* **88**, 1104–1109.
- Redei, G.P.** (1970). *Arabidopsis thaliana* (L.) Heynh: A review of the genetics and biology. *Bibliogr. Genet.* **20**, 1–151.
- Rosbash, M., and Hall, J.C.** (1989). The molecular biology of circadian rhythms. *Neuron* **3**, 387–398.
- Schneider, M., Ow, D.W., and Howell, S.H.** (1990). The in vivo pattern of firefly luciferase expression in transgenic plants. *Plant Mol. Biol.* **14**, 935–947.
- Silverthorne, J., and Tobin, E.M.** (1990). Post-transcriptional regulation of organ-specific expression of individual *rbcS* mRNAs in *Lemna gibba*. *Plant Cell* **2**, 1181–1190.
- Simon, E., Satter, R.L., and Galston, A.W.** (1976). Circadian rhythmicity in excised *Samanea pulvini*. II. Resetting the clock by phytochrome conversion. *Plant Physiol.* **58**, 421–425.
- Stayton, M.M., Brosio, P., and Dunsmuir, P.** (1989). Photosynthetic genes of *Petunia* (Mitchell) are differentially expressed during the diurnal cycle. *Plant Physiol.* **89**, 776–782.
- Sweeney, B.M.** (1987). *Rhythmic Phenomena in Plants*. (San Diego: Academic Press).
- Taylor, W.C.** (1989). Transcriptional regulation by a circadian rhythm. *Plant Cell* **1**, 259–264.
- Uhl, G.R., and Reppert, S.M.** (1986). Suprachiasmatic nucleus vasopressin messenger RNA: Circadian variation in normal and Brattleboro rats. *Science* **232**, 390–393.
- Walling, L., Drews, G.N., and Goldberg, R.B.** (1986). Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. *Proc. Natl. Acad. Sci. USA* **83**, 2123–2127.
- Wuarin, J., and Schibler, U.** (1990). Expression of the liver-enriched transcriptional activator protein DBP follows a stringent circadian rhythm. *Cell* **63**, 1257–1266.
- Young, M.W., Bargiello, T.A., Baylies, M.K., Saez, L., and Spray, D.C.** (1989). Molecular biology of the *Drosophila* clock. In *Neuronal and Cellular Oscillators*, J.W. Jacklet, ed (New York: Marcel Dekker), pp. 529–542.
- Zerr, D.M., Hall, J.C., Rosbash, M., and Siwicki, K.K.** (1990). Circadian fluctuations of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurosci.* **10**, 2749–2762.