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A Novel Circadian Phenotype Based on Firefly Luciferase Expression in Transgenic Plants

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A 320-bp fragment of the Arabidopsis *cab*2 promoter is sufficient to mediate transcriptional regulation by both phytochrome and the circadian clock. We fused this promoter fragment to the firefly luciferase (*Luc*) gene to create a real-time reporter for regulated gene expression in intact plants. *Cab*2::*Luc* transcript accumulated in the expected patterns and luciferase activity was closely correlated to *cab*2::*Luc* mRNA abundance in both etiolated and green seedlings. The concentration of the bulk of luciferase protein did not reflect these patterns but maintained a relatively constant level, implying that a post-translational mechanism(s) leads to the high-amplitude regulation of luciferase activity. We used a low-light video imaging system to establish that luciferase bioluminescence in vivo accurately reports the temporal and spatial regulation of *cab*2 transcription in single seedlings. The unique qualities of the firefly luciferase system allowed us to monitor regulated gene expression in real time in individual multicellular organisms. This noninvasive marker for temporal regulation at the molecular level constitutes a circadian phenotype, which may be used to isolate mutants in the circadian clock.

INTRODUCTION

Light is important to plant cells as a source of energy, as a developmental signal, and as a timing cue. For example, in dark-grown angiosperm seedlings, the light signal initiates a developmental transition known as photomorphogenesis, which permits the etiolated seedling to switch from heterotrophy to autotrophy (Kendrick and Kronenberg, 1986). The best-characterized plant photoreceptor, responsible for many aspects of this transformation, is phytochrome (Quail, 1991). Activation of phytochrome alone, by illumination of plants with red light, initiates many of the photomorphogenic processes from morphological changes (such as the inhibition of hypocotyl elongation) to the molecular events underlying chloroplast biogenesis (such as the expression of the *cab* genes, which encode the chlorophyll *a/b* binding proteins of the light-harvesting complexes).

The energy source for plants in nature, sunlight, varies in intensity and in quality over the diurnal cycle. If plants are experimentally removed from light/dark (LD) cycles and placed under constant environmental conditions, processes at many levels continue to function rhythmically, with periodicities close to 24 hr: this is the hallmark of regulation by the circadian clock (Sweeney, 1987). This endogenous oscillator controls plant functions ranging from stem elongation (Lecharny et al., 1990) and the movements of petals (Engelmann and Johnsson, 1978) to the rhythmic expression of several nuclear genes (Lumsden, 1991; reviewed in Kay and Millar, 1992). Some of the activities controlled by the circadian clock are involved in photosynthesis, and their regulation may be interpreted as a preparation for photosynthetic activity during the day (Nagy et al., 1988a; Adamska et al., 1991; Busheva et al., 1991), although this is probably not the only adaptive significance of circadian control in higher plants. Indeed, circadian regulation in plants shares many properties with the circadian rhythms of other organisms, including temperature compensation of the period in constant ("free-running") conditions and phase shifting by environmental stimuli (Edmunds, 1988). The principal resetting stimulus for most circadian systems is light, which ensures synchronization of the endogenous oscillator to the natural day/night cycle (entrainment). Given the similarities in circadian regulation among diverse species, analysis of the molecular organization of such a system should have broad significance.

Several plant species have been of historical importance in the study of circadian rhythms (Sweeney, 1987), although more significant progress has occurred in Drosophila and Neurospora, which are systems amenable to both classical and molecular genetic studies of circadian rhythmicity. Genetic analysis has defined several loci that are involved in regulating the periodicity of circadian rhythms, notably *per* in Drosophila and *frq* in Neurospora, both of which have been cloned (reviewed in Rosbash and Hall, 1989; Dunlap, 1990; Hall, 1990). In addition, circadian control of transcription has been described for various genes (Loros and Dunlap, 1991): in Drosophila, this class includes the *per* gene (Hardin et al.,

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1990). Recent experiments using fusion constructs in transgenic flies suggest that the control of *per* expression at the transcriptional and/or post-transcriptional levels may be involved in the mechanism of the circadian oscillator (Zwiebel et al., 1991). Thus, analysis of the mechanisms of circadian transcriptional regulation may throw new light on the mechanism of the oscillator.

In higher plants, the *cab* genes have long provided a model system to study phytochrome regulation (Nagy et al., 1988b), but under various conditions the direct light regulation mediated by this photoreceptor is modulated by the circadian clock (Nagy et al., 1988a; Tavladoraki et al., 1989). Studies in etiolated pea (Gallagher et al., 1985) and tobacco (Paulsen and Bogorad, 1988) that were transferred to constant white light showed an initial, brief peak (at ca. 4 hr) and a second, broader peak (ca. 20 hr) of *cab* gene transcription and mRNA abundance, respectively. A very similar pattern was observed after brief red light treatments of etiolated tobacco (Wehmeyer et al., 1990) and bean (Tavladoraki et al., 1989). In light-grown plants under LD conditions (reviewed in Kay and Millar, 1992), the abundance of *cab* transcripts is generally highest in the late morning and falls to a trough in the night. The rise in transcript abundance begins several hours before dawn, indicating the light independence of this expression pattern. Rhythmicity persists when green plants are transferred to constant light (LL) or constant darkness (DD). Rapid reduction of peak levels is observed in DD, although the rate of damping varies among species. The majority of the analyses mentioned above employed probes detecting the sum of all mRNAs from all genes of the PSII *cab* type I family (for a review of the *cab* gene family, see Green et al., 1991). This approach cannot discern any differences in regulation among family members, which may be considerable (Millar and Kay, 1991). The identification of *cis*-acting elements from *cab* promoters, in wheat (Nagy et al., 1988a; Fejes et al., 1990) and Arabidopsis (Millar and Kay, 1991), now permits molecular analyses of the mechanisms mediating circadian responsive transcription of specific genes.

One of the principal limitations of chronobiology at the molecular level has been the use of mRNA markers, which are relatively time-consuming to assay and involve destruction of the experimental material. Molecular genetic approaches would be greatly expedited and classical genetics made possible if a facile, noninvasive assay for promoter activity were employed. The luciferases are the most versatile class of noninvasive reporter genes currently available (Alam and Cook, 1990). Luciferase coding regions from several organisms have been used as reporter genes, although the firefly luciferase has been the most common choice for the analysis of promoter function in eukaryotic systems (Alam and Cook, 1990; Aflalo, 1991; Wood, 1991a). The firefly enzyme catalyzes the oxidative decarboxylation of beetle luciferin using O_2 and Mg^{2+}-ATP as substrates. In vitro at least, coenzyme A (CoA) may also participate in the reaction as a cofactor (Wood, 1991b). A photon is released at 560 nm in 90% of catalytic cycles: this light emission can be quantified with high sensitivity, most commonly in a luminometer. In mammalian cell cultures, the half-life of luciferase activity is approximately 3 hr (Nguyen et al., 1989; Thompson et al., 1991), such that decreases in luciferase synthesis over time, which would not be detectable using more stable reporters such as CAT, may be recapitulated by luminescence. Extremely sensitive video cameras are now available to detect in vivo bioluminescence noninvasively (Wick, 1989; Robinson, 1991); however, the unique ability of luciferase to reveal the temporal regulation of transcription in a single individual has not yet been exploited in any multicellular organism.

We show here that bioluminescence in vivo faithfully recapitulates the phytochrome and circadian regulation of a *cab* promoter fragment fused to the firefly luciferase gene in both green and etiolated tobacco seedlings. Bioluminescence is restricted to the cotyledons of *cab2::Luc* seedlings, confirming that luciferase accurately reports the spatial pattern of *cab* expression. Furthermore, we are able to demonstrate that circadian regulation of basal *cab* transcription can occur in the absence of phytochrome activation.

RESULTS

Cab2::Luc Fusions Reflect Phytochrome Regulation in Etiolated Tobacco Seedlings

Karlin-Neumann et al. (1988) showed that the abundance of the native *cab2* transcript is regulated by phytochrome in etiolated Arabidopsis seedlings. An and coworkers have also shown that a small fragment of the *cab3* promoter (positions −111 to −21), which is 85% identical to the corresponding region of *cab2*, mediates shoot-specific and white light–inducible transcription in transgenic tobacco (Mitra et al., 1989). These studies did not address the induction of circadian rhythmicity in etiolated seedlings, nor were the constructs in tobacco tested with red light. Our earlier studies demonstrated that a fragment of the Arabidopsis *cab2* promoter from position −319 to +3 was sufficient to mediate circadian transcriptional regulation in transgenic tobacco (Millar and Kay, 1991). We therefore used the −319 *cab2* promoter fragment fused to the firefly luciferase gene (*Luc*) to test the phytochrome regulation of this promoter in etiolated tobacco plants and the involvement of the circadian clock in this regulation.

Etiolated tobacco seedlings carrying the *cab2::Luc* fusion gene were harvested at 4-hr intervals after a 2-min exposure to red light. Figure 1A shows the results of a typical RNA gel blot analysis of total RNA extracted from these plants. The induction of the *cab2::Luc* transcript closely parallels that of the endogenous tobacco *cab* gene family (Figure 1A). At this level of time resolution, the initial peak, which occurs 4 hr after the flash, is not distinguishable from that of the endogenous *cab2* mRNA in Arabidopsis (Karlin-Neumann et al., 1988). The first,

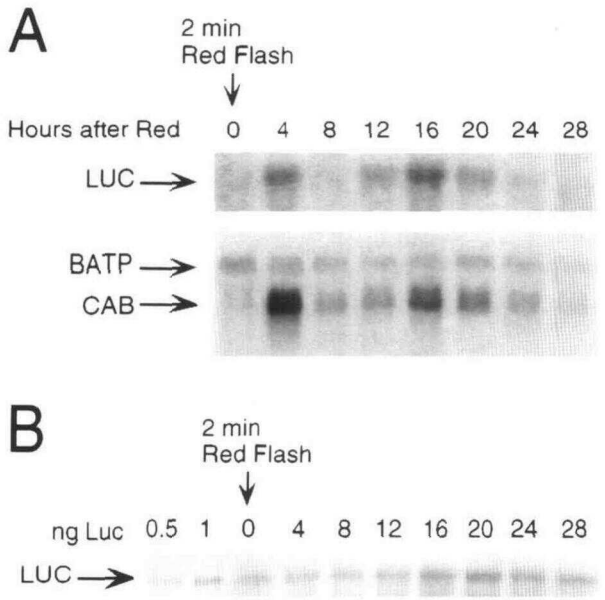

Figure 1. Analysis of *cab2::Luc* Expression in Etiolated Seedlings after a Red Flash.

(A) RNA gel blot analysis. Total RNA prepared from plants harvested at the times indicated following a 2-min red flash was hybridized to coding region probes from *Luc* (LUC), the β subunit of mitochondrial ATPase (BATP), and *cab* (CAB). Twenty micrograms of total RNA was loaded in each lane.

(B) Protein gel blot analysis. Total protein prepared from plants harvested at the times indicated following a 2-min red flash was incubated with luciferase antiserum. Eighty micrograms of total protein was loaded in each lane. 0.5 and 1 refer to 0.5 and 1 ng of purified luciferase (ng Luc).

narrow peak (4 hr) and the second, broader peak (16 hr) of mRNA abundance are very similar to the induction pattern observed by Wehmeyer et al. (1990) for mRNA of the tobacco *cab* family. The first peak of *cab2::Luc* mRNA abundance, as determined by densitometry, shows a fivefold amplitude (comparing 0 hr and 4 hr), as against 10- to 15-fold for the *cab* family, and the second peak a fivefold to sevenfold increase (0 hr versus 16 hr) for both *Luc* and *cab*. In contrast, the abundance of mRNA encoding the β subunit of mitochondrial ATPase assayed on the same blot declined slightly over the course of the experiment. The utility of any reporter of circadian-regulated transcription depends on the activity of the reporter being relatively unstable. The rapid decrease in *cab2::Luc* transcript abundance (fivefold to eightfold from 4 hr to 8 hr) demonstrates that the fusion transcript is sufficiently unstable to follow the fluctuations in transcription rate from the *cab2* promoter.

We next tested the regulation of luciferase protein concentration under the same conditions. Figure 1B shows the results of a typical gel blot of total protein extracted from etiolated *cab2::Luc* seedlings after a red light flash and probed with anti-luciferase antiserum. At the level of luciferase protein, the initial induction is not readily discernable. The second peak, which coincides with the peak of *cab2::Luc* transcript, shows only a slight decrease at 28 hr. Furthermore, the concentration of luciferase protein increases considerably less than the *cab2::Luc* mRNA (approximately threefold, relative to luciferase standards on the same blot, data not shown), with a maximum concentration of approximately 2 ng luciferase per 80 μg total protein. These results suggest that a post-transcriptional mechanism may also affect the accumulation of luciferase protein.

From these results, we conclude that the bulk luciferase protein pool does not reflect the abundance of *cab2::Luc* mRNA; the use of bioluminescence as a marker, however, depends on the activity of the luciferase enzyme rather than the total protein concentration. We therefore used a luminometer to assay for luciferase activity in crude extracts from the tissue samples used in Figure 1B. Figure 2A shows the results of these in vitro luciferase assays. The luminometer data (in arbitrary Light Units) has been calibrated using purified luciferase standards for ease of comparison to data from other luminometers. In sharp contrast to the pattern of luciferase protein accumulation, the specific luciferase activity follows the pattern of mRNA induction closely. Both peaks of luciferase activity coincide with the peaks of mRNA abundance (4 hr and 16 to 20 hr), and the amplitude of the first peak (fivefold from 0 hr to 4 hr) is faithfully maintained. The amplitude of the second peak, however, is 15-fold (from 0 hr to 16 hr) at the level of activity, whereas the abundance of *cab2::Luc* transcript increases only fivefold to sevenfold. This amplification of the luciferase response may be due to the relatively long period of luciferase accumulation during the rise of the second peak; the first peak may be too brief to allow such amplification. Also, luciferase activity does not reflect the fivefold to eightfold decrease in *cab2::Luc* transcript abundance from the fourth to the eighth hour but rather shows only a slight decline in this interval. The maximal activity, 16 hr after the flash, is equivalent to 2 to 3 pg purified luciferase per microgram of total protein, a concentration about 10-fold lower than that of luciferase protein estimated from Figure 1B: this observation is consistent with a post-translational regulation of luciferase activity in intact plant tissue. The rapid decline in activity after the second peak (sevenfold, from 20 hr to 28 hr) indicates that luciferase activity is sufficiently unstable to monitor both increases and decreases in *cab* promoter activity over a timescale of several hours.

Luciferase activity in dark control plants shows a fivefold fluctuation with a peak that coincides with the second peak of red light-induced activity but reaches only 20% of the induced level. The pattern is consistent with a period length greater than 20 hr. We have observed the dark cycle in repeated experiments, although neither the time of seed germination nor the time of first luciferin application affects the phase of the rhythm (data not shown). The peak of emission of firefly luciferase is 560 nm, to which phytochrome is relatively insensitive, so that luciferin application would not be expected to activate phytochrome

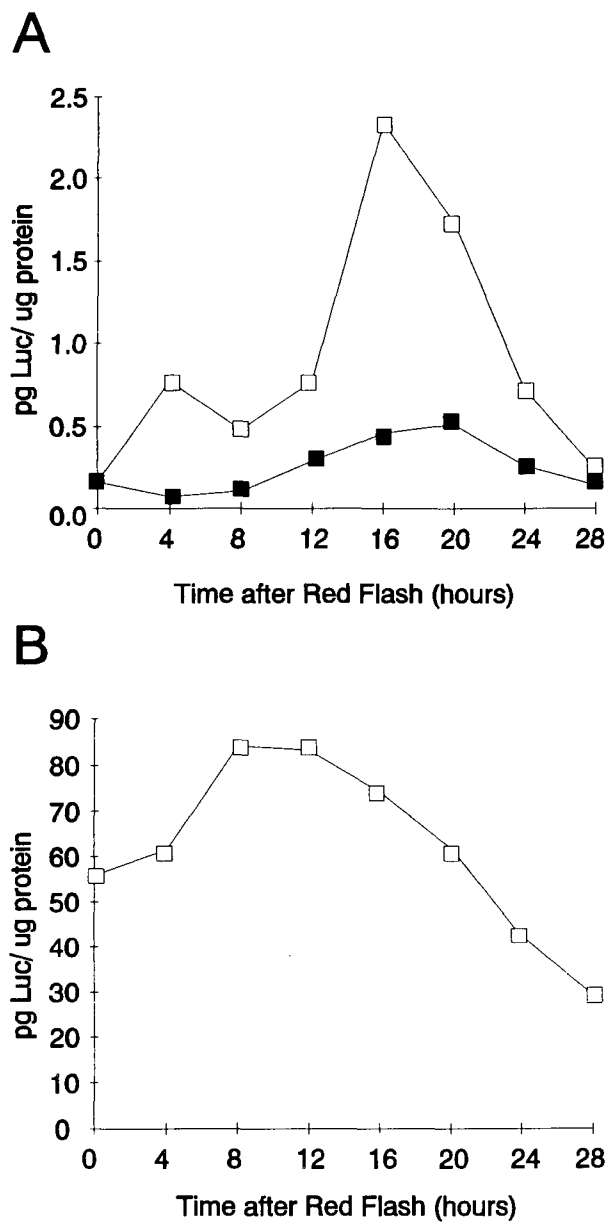

Figure 2. Luciferase Activity in Extracts of Etiolated Seedlings after a Red Flash.

(A) *cab2::Luc* seedlings.

(B) 35S::*Luc* seedlings.

Total protein prepared from plants harvested at the times indicated, following a 2-min red flash (open symbols) or without light treatment (filled symbols), was assayed for luciferase activity in a luminometer. Data in **(B)** are from red-flashed seedlings. Raw luminometer data were normalized using purified luciferase standards.

via luciferase luminescence. Etiolated seedlings were grown in a constant-temperature incubator, as many circadian rhythms are reset by temperature shifts (Edmunds, 1988) and cyclic *cab* expression can be induced in etiolated seedlings by cyclic heat shocks (Kloppstech et al., 1991). The possibility of other, uncontrolled environmental influences cannot be excluded. This fluctuation does not affect the kinetics or amplitude of induction, as very similar patterns of luciferase activity are induced by red flashes administered at different times of day (data not shown). Figure 2B shows a high level of luciferase activity in extracts prepared from etiolated tobacco seedlings bearing luciferase fused to the −343 cauliflower mosaic virus (CaMV) 35S promoter. The activity in 35S::*Luc* seedlings rises to a small peak at 8 to 12 hr and then falls threefold over the course of the experiment. In agreement with previous reports (Nagy et al., 1987), this pattern contrasts the accumulation of activity in *cab2::Luc* seedlings. The levels of luciferase activity in Figures 2A and 2B are not directly comparable, as luciferase is expressed in all organs of the CaMV 35S::*Luc* seedlings, whereas *cab2::Luc* is active only in cotyledons (data not shown; Nagy et al., 1986; Benfey et al., 1990).

The luciferase assays, although rapid and easy to perform, are still destructive. We therefore used an extremely sensitive video camera together with a photon-counting image processor (both from Hamamatsu Photonic Systems, Bridgewater, NJ) to quantify luciferase bioluminescence noninvasively in intact seedlings. Figure 3A shows the quantification of the luminescence in populations of etiolated *cab2::Luc* seedlings, with or without a 2-min red flash. The pattern of luminescence in vivo closely replicates the induction of the luciferase activity. This result implies that neither the access of the luciferin substrate, administered by spraying, nor the availability of endogenous ATP and O_2 obscured the temporal pattern of luminescence. Furthermore, a third peak of luminescence occurs about 28 hr after the center of the second peak (20 hr), showing the cyclic regulation of red light-induced transcription. The third peak appears to be damped by 40% relative to the second. Figure 3B shows the high luminescence levels of CaMV 35S::*Luc* seedlings. As indicated by the in vitro assays, luciferase luminescence shows a small peak (less than twofold in amplitude) after red light treatment, followed by a reduction in activity. Depletion of endogenous substrate pools apparently does not modify the temporal pattern of luminescence significantly, even in these strongly expressing plants during a prolonged time course. The luminescence of 35S::*Luc* seedlings without light treatment was also relatively constant (Figure 3B). Furthermore, the images showed luminescence from all organs of the CaMV 35S::*Luc* seedlings, whereas the *cab2::Luc* plants emitted light only from the cotyledons (data not shown).

Cab2::Luc Fusions Reflect Circadian Regulation in Green Tobacco Seedlings

We previously demonstrated circadian regulation conferred by the −319 *cab2* promoter in green tobacco seedlings when fused to *cat* (Millar and Kay, 1991). We therefore investigated the regulation of the *cab2::Luc* fusion transcript in plants grown

under light/dark cycles for 12 days by harvesting seedlings every 4 hr from one 12-hr light/12-hr dark cycle (12L:12D) followed by 24 hr DD. Figure 4A shows the results of an RNA gel blot from a typical experiment, probed with the *Luc* coding region.

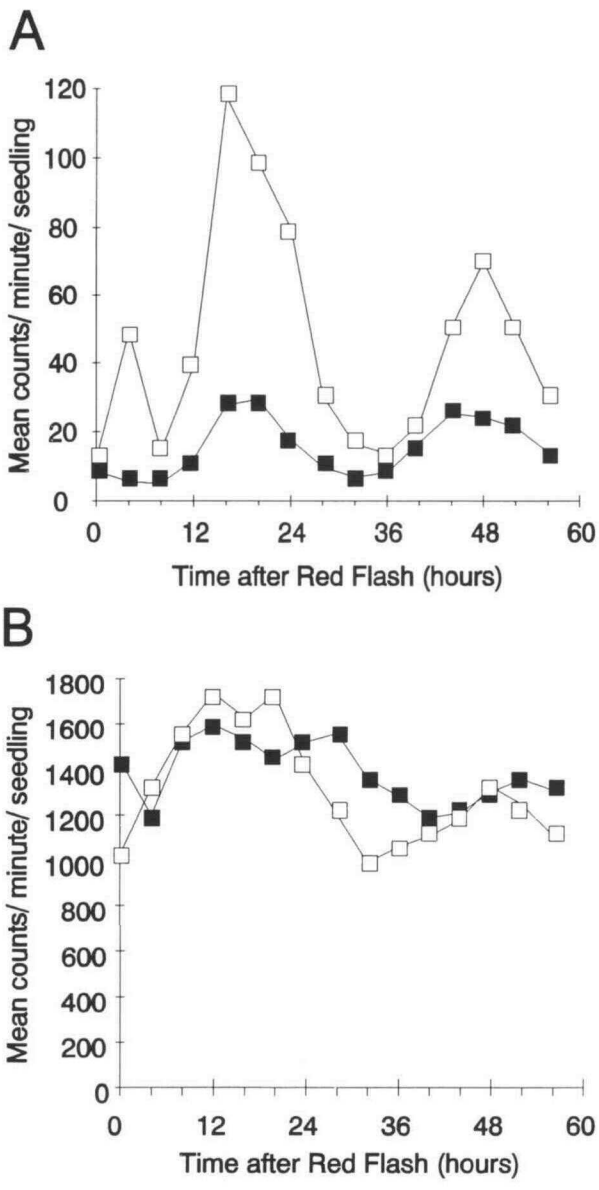

Figure 3. Luciferase Activity in Vivo in Etiolated Seedlings after a Red Flash.

(A) *cab2*::*Luc* seedlings.
(B) 35S::*Luc* seedlings.
Seedlings growing in tissue culture dishes were imaged in a low-light video system at the times indicated following a 2-min red flash (open symbols) or without light treatment (filled symbols). Light emission was quantified using image processing software. Exposure times were 10 min in **(A)** and 2 min in **(B)**. Counts are not directly comparable between **(A)** and **(B)**, as it was necessary to reduce the intensifier gain in **(B)**.

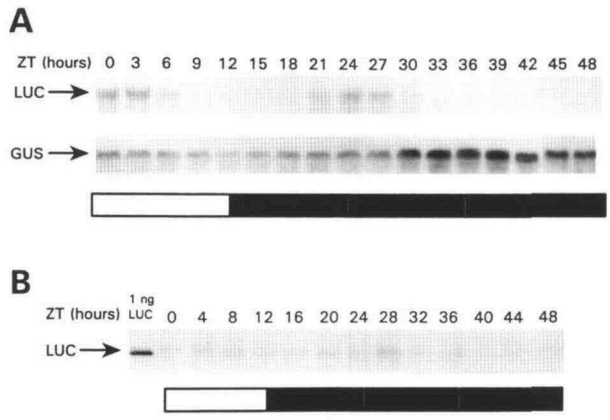

Figure 4. Analysis of *cab2*::*Luc* Expression in Green Seedlings.

(A) RNA gel blot analysis. Total RNA prepared from plants harvested at the times indicated was hybridized to coding region probes from *Luc* (LUC) and GUS. Twenty micrograms of total RNA was loaded in each lane.
(B) Protein gel blot analysis. Total protein prepared from plants harvested at the times indicated was incubated with luciferase antiserum. Twenty micrograms of total protein was loaded in each lane. 1 ng LUC, 1 ng of purified luciferase.
Open box, light period; filled box, dark period.

The blot was rehybridized with a GUS probe to measure the transcript produced from the CaMV 35S::GUS fusion present on the transforming T-DNA. Times are expressed in hours as Zeitgeber time (ZT), which is simply the time since the onset of illumination (Zerr et al., 1990). Zeitgeber is a common term used in chronobiology for the environmental signals that reset the circadian clock. ZT is used to allow comparison of experiments conducted under different LD regimes.

The circadian rhythm of *cab2*::*Luc* transcript abundance is similar to that of the *cab2*::*cat* fusion reported previously (Millar and Kay, 1991), with maxima at ZT0-3 in LD and ZT24-27 in DD, minima at ZT15 and ZT42, and a 15-fold amplitude, as determined by densitometry. The lower amplitude (15-fold versus 50-fold) of the cycle suggests that the *cab2*::*Luc* transcript may be slightly more stable than the *cab2*::*cat* fusion transcript. The peak in DD is damped by 30% relative to the peak in LD. The 35S::GUS transcript shows a contrasting pattern of abundance, with a diurnal fluctuation of low amplitude (twofold to threefold) followed by a plateau in DD. Figure 4B shows the results of a typical gel blot of total protein extracts derived from plants harvested over the time course described for Figure 4A. The concentration of luciferase protein fluctuates with only a low amplitude at close to 1 ng of luciferase per 20 μg total protein in both LD and DD. As in etiolated tissue (Figure 1B), this suggests a post-transcriptional regulation of luciferase accumulation.

The concentration of luciferase activity showed close correspondence to mRNA abundance. Figure 5A shows the luciferase activity in extracts of the samples used for protein gel blotting (Figure 4B) and in samples harvested

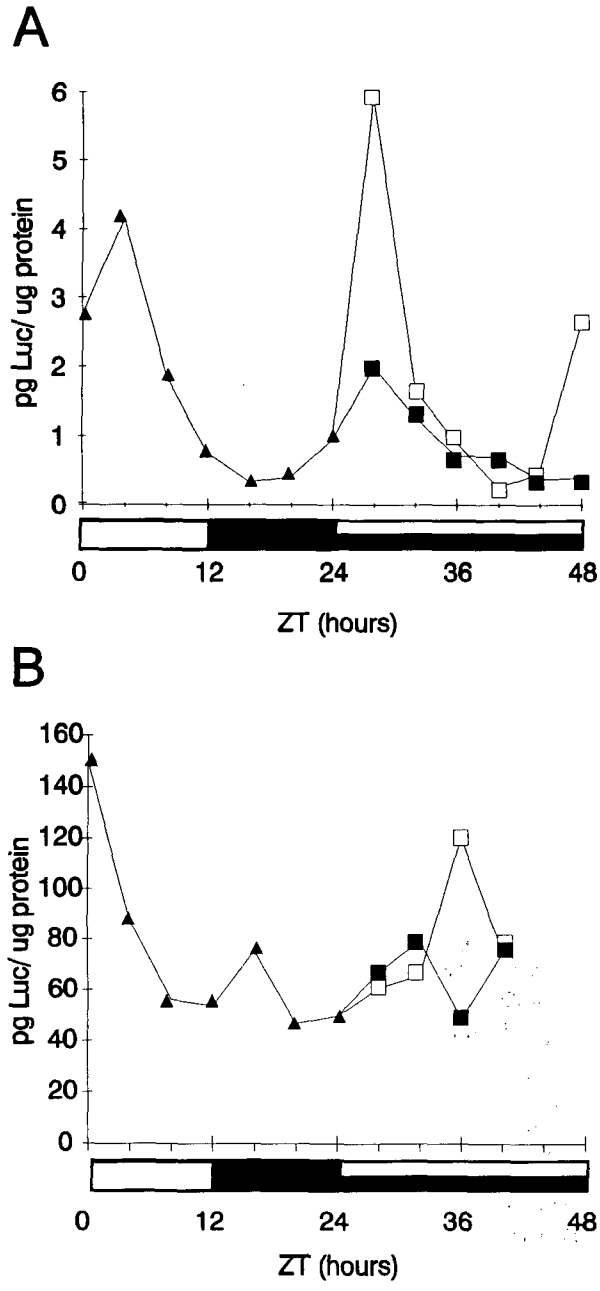

Figure 5. Luciferase Activity in Extracts of Green Seedlings.

(A) *cab2::Luc* seedlings.

(B) 35S::*Luc* seedlings.

Seedlings were transferred from LD (filled triangles) to LL (open squares) or DD (filled squares). Total protein prepared from plants harvested at the times indicated was assayed for luciferase activity in a luminometer. Raw luminometer data were normalized using purified luciferase standards. Open box, light period; filled box, dark period.

simultaneously from plants transferred to LL instead of DD. Luciferase activity follows a clear circadian cycle, with well-defined peaks at ZT4 and ZT28, a minimum at ZT16-20 and an amplitude greater than 10-fold in LD (ZT4 to ZT16). The first peak of activity in DD is damped by 50% relative to LD, and activity thereafter falls continuously from ZT28 to ZT48. In contrast, luciferase activity in LL rises sharply at ZT48, suggesting a second peak 24 hr after the first. Thus, in green tissue also, luciferase activity follows the cyclic accumulation of *cab2::Luc* mRNA very closely. However, the maximal levels of luciferase activity are equivalent to 6 pg of purified luciferase per microgram of protein, approximately 10-fold less than the protein concentration estimated from Figure 4B. Although the addition of extracts from untransformed plants has no effect on the light emission of purified luciferase, the quantum yield of luciferase synthesized in plants has not been directly measured. Nevertheless, these results imply that the pool of active luciferase fluctuates independently of the bulk of luciferase protein. The CaMV 35S::*Luc* lines were again used for comparison to *cab*. Figure 5B shows the luciferase activity from extracts of 35S::*Luc* plants harvested simultaneously with those described for Figure 5A; a threefold reduction from ZT0 to ZT12 is consistently observed in LD in repeated experiments (data not shown). The abundance of the 35S::GUS transcript (Figure 4A) follows a very similar pattern in LD, suggesting that the 35S promoter may show a weak diurnal rhythm in transcriptional activity. The plateau of GUS mRNA in DD, however, is not reflected in luciferase activity from 35S::*Luc*; rather, luciferase activity in both DD and LL shows further fluctuations from ZT24 to ZT40. The plateau of GUS mRNA has not been consistently observed in experiments using this 35S::GUS reference construct (Millar and Kay, 1991; also, data not shown).

The low-light video imaging system confirmed that bioluminescence can act as a novel circadian phenotype. Figure 6A shows the quantification of luciferase luminescence in a typical experiment, using tobacco seedlings grown under LD for 9 days and imaged at the times shown. Figure 6B shows the patterns of luminescence in seedlings that were transferred to constant conditions at ZT12. The patterns of luminescence in vivo closely match luciferase activity determined in extracts, with peaks at ZT4, 28, and 52 and troughs around ZT20 and 44. The amplitudes of cycles from ZT20 to ZT4 (or ZT28) are greater than 10-fold in LD (or LL), whereas the damping of the first peak leads to only a threefold amplitude in DD. The second peak in DD is severely damped, such that the increase in emission from ZT44 to ZT52 is not statistically significant. The patterns of luminescence from 35S::*Luc* seedlings, imaged immediately before the *cab2::Luc* seedlings at each time point, are shown in Figure 6C. The cycle in LD is consistently observed in repeated experiments, with amplitudes between twofold and threefold, consonant with the luciferase activity data from extracts (Figure 5B). The peaks of 35S::*Luc* activity, however, precede those of *cab2::Luc* by approximately 4 hr. Luminescence from 35S::*Luc* in DD shows a threefold to fourfold decrease over the course of the experiment, possibly

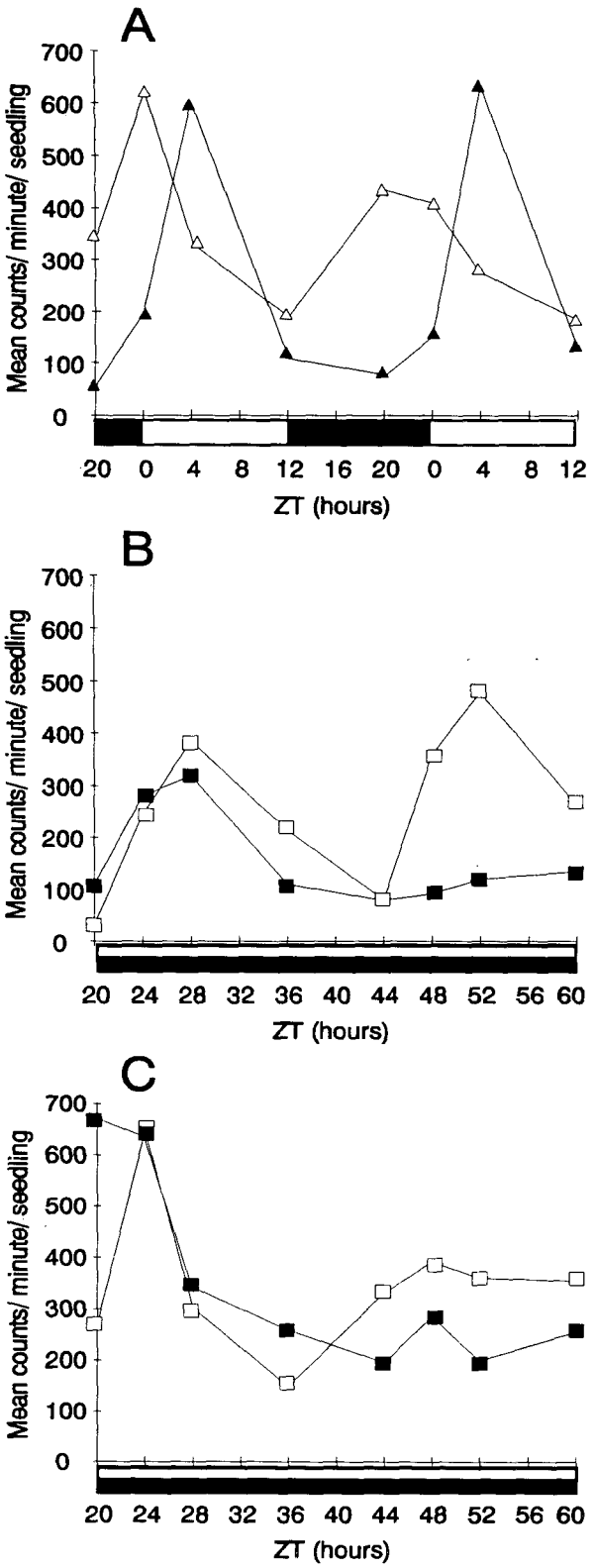

Figure 6. Luciferase Activity in Vivo in Green Seedlings.

(A) *cab*2::*Luc* and 35S::*Luc* seedlings in LD.
(B) *cab*2::*Luc* seedlings in constant conditions.
(C) 35S::*Luc* seedlings in constant conditions.
Seedlings growing in tissue culture dishes were transferred at ZT12 from LD to LL (open squares) or DD (filled squares), or maintained in LD (triangles), and imaged in a low-light video system at the times indicated. Filled triangles, *cab*; open triangles, 35S; open box, light period; filled box, dark period. Light emission was quantified using image processing software. Counts are not directly comparable between *cab*2::*Luc* and 35S::*Luc*, as it was necessary to reduce the intensifier gain for 35S::*Luc*.

indicating a slight substrate limitation in prolonged dark adaptation.

Figure 7 shows a composite of bioluminescence image data from the experiment described in Figures 6A and 6B: tissue culture dishes containing 30 to 35 tobacco seedlings are shown, the first dish during one trough and peak in LD and the second dish over two cycles in DD. The high amplitude of cycling in LD and the first peak in DD are clearly visible by eye, comparing the left- and right-hand panels in the upper two rows; the damping typical of DD conditions is reflected in the decreasing luminescence of the panels descending the right-hand column. We assessed the variability in luminescence between individual seedlings by quantifying the intensity of all the distinguishable single seedlings in images similar to those shown in Figure 7. The standard error of the mean (SE) for peak luminescence in LD, LL, or DD is 9 to 12% of the mean, but a higher SE was observed in the trough (up to 20% of the mean). This variability is due in part to differences in the time of seed germination: when the plates from DD were closely examined, the seedlings that remained brightest in the night phases were found to be significantly younger than the rest of the population. Populations from which these younger seedlings were removed before the experiment began gave an SE for both peak and trough luminescence of 8 to 12% of the mean (data not shown).

*Cab*2::*Luc* Fusions Reflect Organ-Specific Regulation

We tested the distribution of luminescence among the organs of *cab*2::*Luc* and CaMV 35S::*Luc* seedlings to confirm the fidelity of the luciferase marker at a higher level of spatial resolution. The differences in expression pattern between the 35S and *cab* promoters are distinguishable in plates of seedlings imaged with a wide-angle lens; Figure 7 is typical of the *cab*2::*Luc* seedlings. Figure 8 shows the patterns more clearly, at a higher magnification. The cotyledon-specific expression typical of *cab*2::*Luc* and the widespread expression of 35S::*Luc* are consistent with data from other markers (Mitra et al., 1989; Benfey et al., 1990; Chang and Walling, 1992). This result implies that most, if not all, tissues are permeable to luciferin at this stage in development, so the low luminescence intensity observed in the roots of the *cab*2::*Luc* plant is due to low

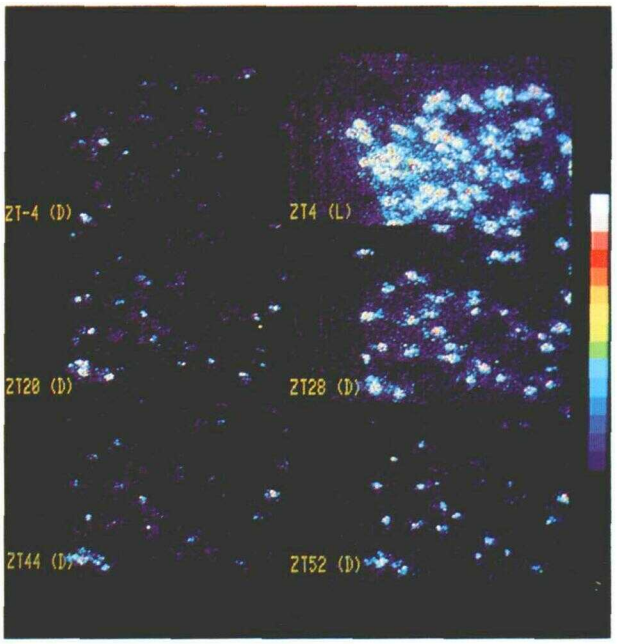

Figure 7. Images of Luminescence from *cab2*::*Luc* Seedlings in LD and DD.

Seedlings growing in tissue culture dishes were imaged for 7.5 min in a low-light video system at the times indicated. Background counts were removed by a noise filter after all images were collected. The upper panels show seedlings at ZT 20 ("−4") and ZT4 in LD; the middle and lower panels show seedlings at ZT20, ZT28, ZT44, and ZT52 in a DD period that began at ZT12. The color scale on the right shows the intensity of luminescence from dark blue (lowest) to white (highest).

luciferase expression. The luminescence per pixel over the cotyledons of the 35S::*Luc* seedling is threefold to fourfold greater than that of the root, whereas the cotyledons of the *cab2*::*Luc* seedling emit 35-fold more than the root. Similar expression patterns have been described for *rbcS*::*Luc* and 35S::*Luc* using contact luminography (a plant pressed flat against film) in older plants (Schneider et al., 1990; Quandt et al., 1992).

DISCUSSION

We have demonstrated in this study that the firefly luciferase gene allows noninvasive, real-time reporting of regulated gene expression. This technique should be broadly applicable to the analysis of transcriptional, post-transcriptional, or translational regulation in any sessile or immobilized organism that is permeable to luciferin and transparent to green light. Furthermore, the instability of luciferase activity allows the bioluminescent marker to recapitulate both increases and decreases in expression over a time course of several hours. We exploited these characteristics to create a novel marker for circadian regulation at the molecular level. Luciferase

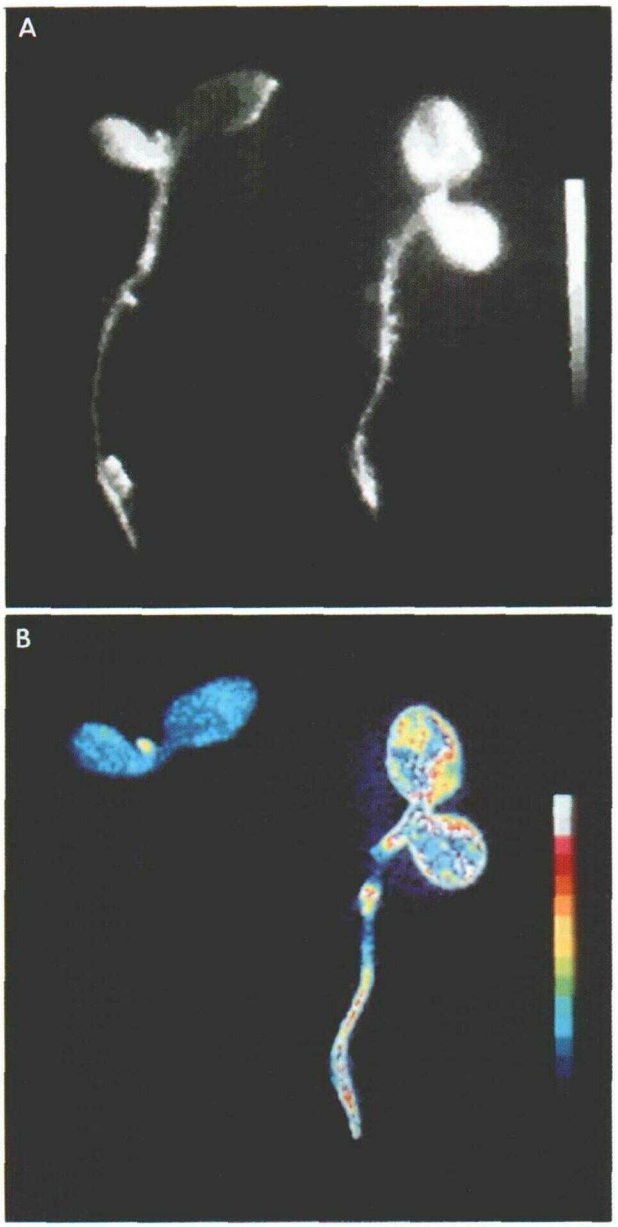

Figure 8. Organ Specificity of Luminescence in *cab2*::*Luc* and 35S::*Luc* Seedlings.

(A) Reference image captured under reflected light.
(B) Luminescence from the same field of view as **(A)**.
Left-hand seedling, *cab2*::*Luc*; right-hand seedling, 35S::*Luc*. Background counts were removed by a noise filter after the image was collected. The color scale on the right of **(B)** shows the intensity of luminescence from dark blue (lowest) to white (highest).

luminescence in vivo reports many of the details of *cab* cycling in etiolated and green, intact seedlings, including the period and phase of free-running rhythms and the damping of peak levels. We expect that this reporter will greatly facilitate studies of the factors influencing the rhythm of transcription from the *cab2* promoter.

A video imaging system is not absolutely required for the noninvasive recording of bioluminescence, as a photomultiplier tube could perform the same task (as in Knight et al., 1991). The spatial resolution of the camera, however, allows us to assay luciferase activity simultaneously in up to several hundred individual seedlings (data not shown). Variability of luminescence is accessible to analysis, even to the level of single, variant individuals in a population: putative mutant seedlings may readily be recovered and grown to seed. The luciferase marker therefore constitutes a circadian phenotype, which should permit a direct genetic screen for clock mutants at the molecular level, without resorting to the brute force approaches used to isolate all currently known clock mutants. Alternatively, organ-level resolution of a few seedlings (Figure 8) and cellular resolution (Gallie et al., 1989) can be achieved using appropriate optics. In this manner, we may investigate the tissue focus of the circadian clock and its characteristics at the cellular level or screen for mutants defective in the spatial regulation of *cab* gene expression.

We used the in vivo luminescence assay to confirm and extend our previous analysis of the circadian regulation of the −319 promoter fragment of Arabidopsis *cab2*. In green tissue, the unambiguous circadian regulation of *cab2*::*Luc* mRNA, luciferase activity, and luminescence in vivo is in close agreement with our previous data on *cab2*::*cat* fusions (Millar and Kay, 1991). The damping in DD is of similar magnitude and transcriptional cycling is maintained in LL, as it is from the wheat *cab1* promoter (Nagy et al., 1988a). In etiolated tissue, the expression patterns we observed are in agreement with previous data for tobacco (Wehmeyer et al., 1990). However, the length of the delay between the inductive red flash and the peaks of luminescence and also the period lengths between peaks deviated considerably from 24 hr. The first peak of luminescence may represent an acute response to the inductive red flash, independent of the circadian system. The subsequent fall in *cab* expression and the appearance of further peaks, in contrast, may be regulated by the circadian clock. Results from studies of *rbcS* transcript levels support this hypothesis: *rbcS* is regulated by a circadian clock in green pea plants (Kloppstech, 1985), and *rbcS* induction by red light is transient in etiolated seedlings (Gallagher et al., 1985). Tomato and tobacco plants, however, show neither the cycling in green tissue (Giuliano et al., 1988; Paulsen and Bogorad, 1988; Piechulla, 1988) nor the transient induction in etiolated tissue (Wehmeyer et al., 1990). The approximately 30-hr period we observed between the second and third peaks in etiolated tissue may be interpreted as a transient (a cycle of deviant period, commonly observed immediately after transfer to free-running conditions; Sweeney, 1987) or as evidence for a difference in period length between etiolated and green tissue. Assuming the clock controlling the second peak of the induced rhythm is similar to that in green tissue, we may infer that the red light pulse sets the clock to about ZT6.

The sensitivity of the luciferase system has allowed us to detect rhythmic activity of the *cab* promoter in populations of etiolated plants grown without red light treatment (Figures 2A and 3A). This result implies that *cab* gene expression in these plants is not only rhythmic but that members of the population are in the same phase, without activation of the phytochrome system. Tavladoraki et al. (1989) have suggested that levels of translatable *cab* mRNA in etiolated bean seedlings may be regulated by a circadian rhythm, although the experimental variability of their assay system prevented a firm conclusion. Neither seed germination nor the first application of luciferin determined the phase of the dark cycle of luciferase activity in preliminary experiments (data not shown), but the possibility of uncontrolled environmental influences currently unknown to us cannot be excluded. Alternatively, these data may be interpreted as evidence for phase determination during seed development; rhythmicity is not unknown in seed, as circadian rhythms of gas exchange have been observed even in dry onion seed (Bryant, 1972). Circadian changes in the spectral properties of phytochrome and in the responsiveness of hypocotyl unhooking to red light have also been described in etiolated zucchini seedlings (Horwitz and Epel, 1978). We did not pursue this line of investigation, as the kinetics and amplitude of the red light response are not affected by the phase of the red light treatment relative to the dark rhythm (data not shown). Possible explanations for the latter observation include the hypothesis that saturation of the phytochrome system either resets the dark cycle to a constant phase or uncouples the *cab* promoter from it, or that the high induced activity masks the low level of the dark rhythm while both continue to operate. The first hypothesis is attractive in that it does not require two circadian clocks, controlling *cab* expression in different conditions, and postulates only an extreme form of the known, phytochrome-mediated phase-shifting (Tavladoraki et al., 1989).

Luciferase enzyme activity, whether it is assayed in extracts or in vivo, follows the pattern of *cab2*::*Luc* mRNA abundance closely (comparing Figure 1A with 2A and 3A, and Figure 4A with 5A and 6A). The major difference between the mRNA and activity patterns is in the relative heights of the first (4 hr) and second (16 to 20 hr) peaks of red light-induced activity. Whereas mRNA abundance reaches comparable levels in these peaks, luciferase activity is higher in the second peak. The prolonged high abundance of *cab2*::*Luc* mRNA in the second peak may allow the unstable luciferase activity to rise further than in the brief, first peak. The use of in vivo bioluminescence as a marker for circadian regulation depends crucially upon the stability of both the luciferase transcript and the luciferase enzyme activity. Our data are not sufficient to determine accurately the half-life of the *cab2*::*Luc* mRNA, the luciferase protein, or the luciferase enzyme activity in vivo. The *cab2*::*Luc* fusion transcript is unstable enough to fall dramatically after accumulation to high levels, both after red light induction and in diurnal and circadian cycles. At the level of protein concentration, in contrast, the amplitude of regulation is diminished in both conditions. This dichotomy indicates that translational or post-translational mechanism(s) mask the fluctuations of mRNA abundance. The luciferase protein may be sufficiently stable in vivo to allow the protein pool to accumulate far beyond the level of the de novo synthesis that is limited to periods of high mRNA abundance.

The post-translational mechanism(s) responsible for the

fluctuations in luciferase activity that we observe despite the relative constancy of the bulk protein concentration is unknown at present. Several examples of enzymatic activities that fluctuate independently of the enzyme abundance have been described in plants (for example, Nimmo et al., 1987). Bacterial luciferase is inactivated in vivo (Tu and Hastings, 1975) in a process (or processes) dependent on the presence of the aldehyde substrate (Baldwin et al., 1978); the inactive enzyme may accumulate to high levels (Mitchell and Hastings, 1970). Consistent with such a mechanism, the high-amplitude variations in luciferase activity observed in Figures 2 and 5 are dependent upon exposure of the plants to luciferin prior to harvesting (data not shown). The stability of firefly luciferase activity in the absence of substrate restricts its utility as a reporter gene in higher plants (Quandt et al., 1992). Further investigation of the mechanisms responsible for the apparent changes in specific luciferase activity will be best approached in a system more amenable to biochemical feeding studies than intact seedlings.

Earlier publications (Subramani and DeLuca, 1988; Koncz et al., 1990) identified the access of luciferin to luciferase in living tissue as a potential problem for the firefly luciferase reporter system, as beetle luciferin is negatively charged at neutral pH, and also because the native firefly luciferase is targeted to the peroxisome (Gould et al., 1990). The close correspondence between the patterns of luciferase activity measured in extracts and in vivo implies that neither the availability of luciferin nor the endogenous pools of ATP and O_2 (and possibly CoA; Wood, 1991b) obscured the cyclic pattern of luminescence in our experiments with *cab*2::*Luc*. In studies using older plant material and promoters expressed in more diverse cell types than *cab*, substrate limitation may influence the patterns of luminescence observed in vivo (Barnes, 1990; Schneider et al., 1990).

We have recently extended our analysis of circadian regulation using the firefly luciferase system to transgenic Arabidopsis, with results similar to those described above for tobacco. We are now pursuing a molecular and genetic approach to isolate novel mutants in the plant circadian system. Such mutants will be essential in elucidating not only the molecular architecture of this biological clock and its interaction with other receptors (notably phytochrome) but also the role of this system in the regulation of metabolism and development in higher plants.

METHODS

Plant Material

Tobacco plants (var. SR1) were maintained in sterile culture on MS (Murashige and Skoog, 1962) medium to provide leaves for transformation experiments. Transformation was performed according to standard techniques (Horsch et al., 1988). Regenerated kanamycin-resistant T1 plants (primary transformants) carrying *cab*2::*Luc* or 35S::*Luc* fusions were grown up in a greenhouse to allow seed set. T2 seed of strongly expressing T1 lines were grown in sterile culture on MS medium containing kanamycin, at 20°C, for 6 to 10 days in 16L8D, then for 2 days in 12L12D (green tissue) or for 7 days, wrapped in aluminium foil, in a constant-temperature incubator in a dark room (etiolated tissue). Red light treatments were for 2 min using the source described (Nagy et al., 1986). Three to four independently transformed lines were analyzed for each construct in each experiment. Plants used for protein or luciferase extracts and for in vivo imaging were sprayed with 5 mM D-luciferin (Analytical Bioluminescence Laboratories, San Diego, CA) in 0.01% Triton X-100 three times before the start of the experimental time course and once approximately 30 min prior to each harvesting or imaging time point.

Clones and Construction

The construction of the −319 to +3 *cab*2 promoter fragment fused to the wheat *cab*1 untranslated RNA leader has been described (Millar and Kay, 1991). This promoter–leader fusion was ligated, at the SalI site used previously in *cab*2:*cat* fusions, to the firefly luciferase coding region from plasmid pJD261, kindly provided by Dr. Jeff deWet (Stanford University) followed by the poly-A addition sequence from pea *rbcS*-E9. The *cab*2::*Luc*::E9 fusion was then transferred to the polylinker of the binary vector pMON721 (Monsanto Corporation, St. Louis, MO) containing a CaMV 35S::GUS::*rbcS*-3C fusion described previously (Millar and Kay, 1991). The *Luc* coding region was also inserted into the CaMV 35S expression vector pMON530E9 (Cuozzo et al., 1987) to create the 35S::*Luc* fusion.

RNA Extraction and Analysis

Total RNA was prepared from whole seedlings and analyzed in RNA gel blots as described previously (Nagy et al., 1988c). The probes employed were from the coding regions of the β subunit of tobacco mitochondrial ATPase (Boutry and Chua, 1985), Arabidopsis *cab*1 (Leutweiler et al., 1986), and firefly luciferase (de Wet et al., 1987). Autoradiograms of RNA gel blots were quantified using a densitometer (Helena Laboratories, Beaumont, TX). Given the fluctuations of the 35S::GUS fusion and β-ATPase transcripts, the amplitude of *cab*2::*Luc* transcript regulation was calculated from the absolute density of the relevant *cab*2::*Luc* bands alone.

Protein Extraction and Analysis

Total protein was extracted from whole seedlings in boiling 2% SDS extraction buffer and analyzed in protein gel blots as described (Harlowe and Lane, 1988). In some experiments, 1 × Cell Lysis Reagent (CLR, containing 1% Triton X-100; Promega) was used with very similar results. Protein content of the samples was determined using the DC protein assay kit (Bio-Rad, Richmond, CA), according to the manufacturer's instructions. Proteins were transferred to supported nitrocellulose membranes (BA-S; Schleicher & Schuell, Keene, NH), and antibody binding was visualized using the Proto-Blot kit (alkaline phosphatase/NBT reaction) as recommended by the manufacturer (Promega). The luciferase antiserum was raised from rabbits immunized with purified firefly luciferase (United States Biochemical Corp. or Boehringer Mannheim).

Luciferase Extraction and Analysis

Total protein was extracted from whole seedlings ground in liquid nitrogen, using a 2:1 volume ratio of ice cold 1 × CLR, supplied as part of a luciferase assay kit (Promega). This method extracts luciferase protein as efficiently as boiling the tissue powder in 2% SDS buffer, as judged by protein gel blots (see above). The extracts were prepared in batches of no more than 20, were cleared by two 5-min centrifugations at 10,000*g*, at 4°C, and were kept on ice at all times as the luciferase activity proved unstable in extracts. Protein content of the samples was determined using the DC protein assay kit (Bio-Rad), according to the manufacturer's instructions. Luciferase activity was measured for 10 2-sec intervals in a luminometer (model No. ILA911; Tropix, Bedford, MA), using 10 μL of extract in 50 μL of Luciferase Assay Reagent (Promega) according to the manufacturer's recommendations. Extracts of 35S::*Luc* plants were diluted 10-fold in 1 × CLR containing 1 mg/mL bovine serum albumin (BSA) and assayed as described. Luminescence from the highest 2-sec count was normalized using a standard curve prepared with serial dilutions of purified firefly luciferase (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 1 × CLR containing 1 mg/mL BSA. In this system, 100 fg of luciferase is routinely detectable (100 RLU/2 sec, threefold above background), luminescence is linearly related to luciferase concentration over at least four orders of magnitude, and the addition of extracts from etiolated or green, untransformed tobacco plants has no effect on the assay (data not shown).

In Vivo Imaging of Luciferase Bioluminescence

Plant materials were grown and sprayed with luciferin as described above. Seedlings were imaged at 20°C in tissue culture dishes using intensified cameras (VIM) and photon-counting image processors (ARGUS-50 or ARGUS-100) purchased from Hamamatsu Photonic Systems (Bridgewater, NJ). Exposure times were 7.5 min for green and 10 min for etiolated *cab2*::*Luc* seedlings, and 2 min for all CaMV 35S::*Luc* seedlings. Due to the strong expression of 35S::*Luc* seedlings and limited intrafield dynamic range of the camera, it was necessary to reduce the gain of the intensifier for the images of these plants. Therefore, the counts for *cab*::*Luc* and 35S::*Luc* plants are not directly comparable. Luminescence was quantified from images captured in centroid-processing mode (this records a single pixel of unit depth for each photoelectron detected by the camera) and corrected for background counts from thermal electrons. Figure 8 was taken using a macro lens, with a 10-min exposure. The images in Figures 7 and 8 were compiled in the ARGUS image processor, passed through a noise filter to remove background counts, and captured by 35-mm photography of the imaging monitor.

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