A circadian clock regulates transcription of the wheat *Cab-1* gene

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In wheat plants grown under 12-hr light (L)/12-hr dark (D) cycles, the expression of a single member of the *Cab* family, designated the *Cab-1* gene, cycles in plants that have been transferred to continuous light or darkness, indicating control by an endogenous circadian clock. The plant photoreceptor, phytochrome, interacts with the clock to govern the level of *Cab-1* RNA. Furthermore, when the wheat *Cab-1* gene is transferred to tobacco, the circadian regulation is maintained. Fusion of the upstream region of the *Cab-1* gene to the bacterial chloramphenicol acetyltransferase (CAT) gene results in the circadian fluctuation of CAT mRNA in transgenic plants. Not only does this indicate that the circadian clock is acting at the transcriptional level, but it also identifies the *cis*-acting element that mediates this response.

[Key Words: Wheat *Cab-1* gene; circadian clock; transcriptional regulation]

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The phenomenon of biological rhythms that control physiological and biochemical events is now widely established in many organisms (for review, see Feldman 1982). In higher plants, many physiological processes respond to an endogenous circadian rhythm (for review, see Vince-Prue 1983) which, although entrained by environmental stimuli (e.g., light), also persist under constant conditions in the laboratory. For example, the leaves of certain legumes, termed nyctinastic species, exhibit a rhythmic upward and downward motion that has a periodicity of ~24 hr (Satter and Galston 1981). When these plants are placed in continuous light or darkness, the rhythm persists for several days with a gradual damping and can be reinitiated with the correct light regime. Despite the progress that has been made in identifying these rhythms in higher plants, very little is known about the underlying molecular mechanisms responsible for this mode of regulation. Therefore, it is not only necessary to identify the genetic loci controlling these physiological processes but also to characterize in detail the rhythmic responses at the biochemical or gene level.

Our laboratory has been interested in the mechanisms by which light regulates plant gene transcription (Kuhlemeier et al. 1987a). One of the genes we have chosen to study is the wheat *Cab-1* gene, which encodes the major light-harvesting chlorophyll-binding protein of the chloroplast (Lamppa et al. 1985a). In wheat, the expression of the *Cab-1* gene is greatly stimulated by light, and when this wheat gene is transferred to tobacco it retains its light sensitivity and tissue specificity in the heterologous nuclear background (Lamppa et al. 1985b). While conducting light-induction experiments, we found that the *Cab-1* transcript level was greatly diminished in plants that had been placed in continuous darkness for 4

days. Upon reillumination of the plants in continuous white light for 24 hr, the *Cab-1* transcript level increased dramatically beyond its original level in the previous light period (Lamppa et al. 1985b). In an attempt to understand this apparent 'superinduction' by light, we have examined the expression of the *Cab-1* gene more closely under different conditions.

We grew wheat plants under 12-hr light (L)/12-hr dark (D) cycles at constant temperature and measured the *Cab-1* transcript abundance at 2-hr intervals throughout the entire cycle. Interestingly, we found a dramatic fluctuation in transcript level, which also persists in constant light and dark. A previous report by Kloppstech (1985) had indicated that the steady-state levels of *rbcS* gene family transcripts in the pea were under circadian control in constant light and that the pea *Cab* genes fluctuated diurnally under light/dark cycles. However, no data were presented as to the mode of regulation, nor was the expression of a single gene investigated. Here, we present our observations on the cycling of the *Cab-1* transcript level and its regulation by the interaction of an endogenous circadian rhythm with the photoreceptor, phytochrome. This complex expression pattern is maintained in transgenic tobacco containing *Cab-1* gene constructs. We demonstrate that *Cab-1* transcript stability is unaffected by the clock, which acts at the transcriptional level.

Results

Cab genes but not *rbcS* genes are controlled by a circadian rhythm

Seven-day-old wheat seedlings were harvested at 2-hr intervals throughout the 12-hr L/D cycles. RNAs were ex-

tracted from the samples and analyzed by slot blots. Figure 1A shows that the transcript level of the wheat *Cab* gene family undergoes dramatic oscillation in the course of the L/D cycles. The transcript level is highest during the day between 8 AM and 12 PM; it declines steadily toward the end of the day and finally becomes undetectable after midnight. Interestingly, the level of the *Cab* family RNA begins to increase before the onset of illumination. By performing dilution experiments, we estimated that there is at least a 20-fold difference in the transcript level between the highest and the lowest point of the L/D cycle. In contrast, the wheat *rbcs* transcript level does not display any obvious oscillation (Fig. 1B).

To see whether the oscillation in the *Cab* gene expression is regulated by a diurnal or a circadian rhythm, we determined the transcript level when the plants were placed in constant darkness for 24 hr. We found that the *Cab* transcript level continues to oscillate in a free-running cycle during the first 12 hr of extended darkness; the increase and decrease of the transcript level parallel those in the light (Fig. 1A). This free-running cycle continues for at least two cycles in the dark, but the amplitude becomes increasingly damped with time until it is no longer recognizable in the third cycle (data not shown). These results indicate that the oscillation in *Cab* gene expression occurs irrespective of the L/D conditions of the environment and is therefore regulated by a circadian rhythm. Control experiments revealed that the *rbcs* transcript level declines steadily during extended darkness (Fig. 1B), exhibiting no rhythmicity.

Figure 1. The level of expression of the *Cab* gene family, but not the *rbcs* gene family, is regulated by both a diurnal and circadian rhythm in wheat. Wheat seedlings were grown under a L/D cycle, and samples were harvested every 2 hr throughout the cycle of the seventh and eighth day. Some of the plants were kept in extended darkness instead of receiving light on the eighth day. RNA was isolated, and duplicate slot blots were made and hybridized with either *Cab-1*- (A) or *rbcs*- (B) coding sequence probes that hybridize to all members of each of the multigene families. Each slot contained 5 μ g RNA. White and black bars indicate periods of light and dark, respectively.

Expression of the Cab-1 gene is controlled by a circadian clock

The experiments in Figure 1 were carried out with a cDNA probe that does not discriminate among members of the *Cab* gene family. Because only total *Cab* transcript level was measured, it could be argued that different *Cab* genes display different expression profiles. To examine this point, we used 5' nuclease S1 protection to assay specifically the wheat *Cab-1* (Lamppa et al. 1985b) transcript levels during the L/D cycles. Figure 2A shows that the *Cab-1* gene indeed exhibits high transcript level during the day and low or undetectable transcript level at night. The cycling of the *Cab-1* transcript level is similar to that observed for the total *Cab* transcript. Moreover, neither the first period of extended darkness nor extended light affects the rhythm, except that in the case of extended darkness, the expression level (Fig. 2B) tends to be lower than that of the control (Fig. 2A). Despite a slight variation in the time of peaking from one experiment to another, it occurs between 8 and 10 AM (Fig. 2A–C). As for the whole gene family, the *Cab-1* gene damps rapidly during a longer free-running cycle in extended darkness (data not shown). Longer exposure of the autoradiograms also demonstrates that the increase in *Cab-1* transcript level precedes the onset of illumination (not shown).

We have also determined the *Cab-1* transcript level of wheat seedlings germinated in the light and kept under constant illumination for 7 days. Even under constant light conditions in which the plants have never seen darkness, the *Cab-1* gene expression cycles with a periodicity of ~ 24 hr (Fig. 2D). These results provide further evidence that the *Cab-1* gene is regulated by an endogenous circadian rhythm, which is independent of the L/D transition. It is possible that the phase of the rhythm under these conditions has been shifted slightly forward, although this is difficult to measure accurately.

The level of Cab-1 gene expression during the cycle is modulated by phytochrome

In the extended darkness experiments of Figure 2, we noticed that although the oscillation of the *Cab-1* gene expression is maintained for two periods, the transcript level decreases with time. These results suggest that under these conditions, the *Cab-1* transcript abundance is also regulated by light. In an attempt to identify the photoreceptor that mediates this effect, we exposed the plants to red or far red light for 30 min at the end of the day. Figure 3C shows that red light increases the transcript abundance over the dark control (Fig. 3B) and restores the level to that obtained with 12-hr white light (Fig. 3A). In contrast, far red light greatly suppresses the *Cab-1* transcript to a level (Fig. 3D) even lower than that in the dark (Fig. 3B). However, the suppressing effect of far red light can be abrogated efficiently by a subsequent illumination with red light (Fig. 3E). The antagonistic effects of red and far red light together provide conclusive evidence that the level of *Cab-1* gene expression is

indeed regulated by light via the photoreceptor phytochrome.

Circadian regulation of the wheat Cab-1 gene is maintained in transgenic tobacco

In an attempt to define *cis*-acting elements of the *Cab-1* gene that mediate the circadian response, we have transferred the *Cab-1* gene and two chimeric derivatives (Fig. 4) into tobacco via Ti-mediated gene transfer techniques.

Remarkably, the circadian rhythm of the wheat *Cab-1* gene can be recapitulated in transgenic tobacco plants grown under L/D cycles (Fig. 4A). Moreover, a similar free-running periodicity of ~24 hr is maintained in transgenic tobacco. Therefore, the mechanisms by which circadian clocks regulate *Cab-1* gene expression must be highly conserved between the divergent orders of monocots and dicots, indicating an important function for this process.

The oscillation of the *Cab-1* transcript during the L/D cycles can be due to transcriptional or post-transcriptional regulations, or both. To ascertain the level of control, we assembled two chimeric genes and examined their expression in transgenic tobacco. Construct B is comprised of a 1.8-kb 5'-upstream fragment of the *Cab-1* gene fused to the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT). This transcription unit is expected to produce a CAT mRNA with 31 nucleotides of the 5'-leader RNA from *Cab-1*. Construct C contains the -124 5'-deletion mutant of *Cab-1*, with a cauliflower mosaic virus (CaMV) 35S enhancer fragment placed at its 3' end. This transcription unit produces *Cab-1* mRNA with its authentic 5' terminus. The constitutive 35S enhancer increases the transcription rate of this mutant, making it easier for transcript detection.

We found that the amount of *Cab-1* mRNA produced by construct C does not change significantly during L/D cycles (Fig. 4C). Therefore, the results also show that the circadian clock does not control *Cab-1* mRNA stability. Moreover, there is no differential stability of the mRNA in the light as compared with the dark. As a further control, we have also investigated the expression of the 35S-CAT construct in transgenic tobacco and find that the CAT mRNA level does not change in L/D cycles (data not shown). In contrast to construct C, construct B exhibits circadian rhythm (Fig. 4B), enabling us to express the bacterial CAT mRNA in a rhythmic pattern in the transgenic plant. Taken together, these results show that circadian control of *Cab-1* gene expression is largely, if not exclusively, a transcriptional phenomenon and that the *cis*-acting element that responds to this regulation resides upstream of -124 of the *Cab-1* gene.

Discussion

Despite the observation in pea (Kloppstech 1985) that the steady-state abundance of *rbcS* transcripts cycle with circadian rhythmicity, it was not known whether a

Figure 2. The wheat *Cab-1* RNA level is regulated by an endogenous circadian oscillator. Wheat plants were grown for 7 days in L/D cycles (A–C) or in continuous white light (D). Identical sets of plants were placed in different light regimes and harvested every 2 hr. (A) A normal L/D, (B) extended darkness, (C) extended light, (D) continued white light. RNA was isolated, and the level of *Cab-1* RNA was determined using a gene-specific single-stranded DNA probe in 5' nuclease S1 protection assays. Each sample contained 25 µg RNA.

circadian clock controlled the expression of the *Cab* gene family. Furthermore, if one is to characterize such a response, it is necessary to select a single gene within the family for study. We show here that the wheat *Cab* gene family, as a whole, and the *Cab-1* gene, in particular, also exhibit cyclic variation in their expression levels. In addition, we found that the cycling persists even under constant environmental conditions, indicating that the *Cab-1* gene expression is under the control of an endogenous circadian clock. The regulatory mechanism of the *Cab-1* clock appears to be conserved between monocots and dicots because circadian control of the wheat *Cab-1* gene can be recapitulated in transgenic tobacco plants. However, the circadian cycling of *rbcS* genes in pea (Kloppstech 1985) is not well conserved, as we could not detect any rhythmicity in the expression of this gene family in wheat.

Although the oscillation of *Cab-1* transcript level continues with a 24-hr periodicity under extended darkness, the amplitude damps with time, indicating that light plays a role in regulating the expression level. We found that the damping in continuous darkness can be prevented by a brief illumination with red light but is exacerbated by far red light. Moreover, the suppressing effect of far red light is reversible by red light, thus implicating phytochrome as the photoreceptor. All of these light treatments affect only the amplitude and do not elicit a phase shift. These results together indicate that phytochrome is involved in regulating the level of *Cab-1* transcript within the periodicity of the clock. The interplay between phytochrome and the circadian clock is reminiscent of a similar interaction reported for higher plant photoperiodism (Vince-Prue 1983) and nyctinastic leaf movements in some legumes (Simon et al. 1976; Satter et al. 1977). However, it does differ in the fact that phytochrome is exerting a quantitative effect on the *Cab-1* gene, whereas in the physiological systems it also affects the clock directly by altering the periodicity and phase. It is not surprising that the circadian rhythm that regulates transcription of the *Cab-1* gene differs in some respects from the known morphological rhythms, as this is the first time such a phenomenon has been studied at the molecular level. At this level of resolution, phytochrome does not appear to phase-shift the cycle, and this discrepancy may be due to different biological clocks controlling the different processes. The precise mechanism of the interaction between the clock and phytochrome is still unknown, but our finding that the wheat *Cab-1* transcript level is under such control pro-

vides the first step toward resolving the clock in molecular detail.

Many physiological processes and biochemical reactions, ranging from conidiation in *Neurospora* (Feldman 1982) to eclosion in *Drosophila* (Hall and Rosbash 1987), are known to be controlled by circadian rhythms. The

Figure 3. Phytochrome interacts with the circadian clock to control the level of expression of the *Cab-1* gene within the rhythm. Wheat plants were grown for 7 days, as described in Figures 1 and 2. As a control, some plants were harvested during a normal L/D cycle (A) or extended darkness (B) during the eighth day. The rest of the plants were given different red (R)/far red (FR) light treatments at 8 PM, as indicated (see Methods), followed by extended darkness during which plants were harvested and assayed for *Cab-1* transcript level, as described in Figure 2. Each sample contained 25 μ g RNA.

Figure 4. The circadian control of expression of the wheat *Cab-1* gene is maintained in transgenic tobacco and exerted at the transcriptional level. (A) The wheat *Cab-1* gene extending from -1.8 to $+1.1$ kb was transferred to tobacco plants, and the expression of the *trans* gene was monitored during L/D cycles by 5' nuclease S1 protection assays. (B) The expression of a *Cab*-CAT-E9 construct was analyzed in transgenic tobacco during both L/D cycles, as well as extended darkness, by 3' nuclease S1 protection assays. (C) A hybrid construct consisting of the *Cab-1* gene from -124 to $+1100$ with the CaMV 35S enhancer fused at the 3' end was transferred to tobacco. The expression of this *trans* gene during L/D cycles was assayed by 5' nuclease S1 protection assays, as it retains the same start site as the authentic *Cab-1* gene. Samples of 25 and 50 μ g RNA were used for 5' and 3' nuclease S1 analyses, respectively.

mode of control, however, has been studied only in a limited number of cases. For example, Dunlap and Hastings (1981) have shown that the cyclic variation of the luciferase activity in *Gonyaulax* is due to circadian-controlled synthesis and degradation of the luciferase protein. In *Drosophila*, Reddy et al. (1984) reported that a 1-kb transcript undergoes dramatic circadian oscillation, but it is not known whether the biological clock controls transcription rate or transcript stability, or both. We have addressed this question with respect to the wheat *Cab-1* gene by analyzing the expression of chimeric *Cab-1* genes in transgenic tobacco. These experiments have established firmly that the circadian clock controls *Cab-1* gene expression at the transcrip-

tional level and that transcript stability is not affected. The *cis*-acting element that mediates the clock regulation is located between -1800 and -124 , 5' upstream of the *Cab-1* gene. This is the first time that a clock has been shown to function at the level of transcription and provides a novel opportunity for working upward from the response of the *Cab-1 cis* elements toward identifying the other components that constitute this rhythmic response.

Previous work has demonstrated that the *Cab* genes of higher plants are regulated by phytochrome in etiolated seedlings (cf. Mosinger et al. 1985; Tobin and Silverthorne 1985; Nagy et al. 1986) and induced by white light in mature, green plants grown under L/D diurnal cycles (Lamppa et al. 1985a,b). We have performed light-induction experiments with transgenic tobacco plants containing the *Cab-1* gene and found that upon reillumination of dark-adapted plants, the transcript level is higher than that of plants before dark adaptation (Lamppa et al. 1985b). This superinduction is most likely due to the dramatic variation in *Cab-1* transcript levels at different times of the light period (Figs. 2 and 4). The same phenomenon may account for, at least in part, the large variation of *Cab* gene expression levels detected among independent transgenic petunia plants, especially if samples harvested at different times of the day were compared (Jones et al. 1985). Thus, by considering the expression of the *Cab-1* gene within the context of a circadian clock, we can now account for the superinduction of this gene observed previously.

Although white light induction of *Cab* gene expression in mature, green plants has been known for some time (cf. Tobin and Silverthorne 1985; Kuhlemeier et al. 1987a), the photoreceptor for this response has not yet been identified. We show here that the photoreceptor is phytochrome. However, the action of phytochrome appears to be secondary because it can be overridden by the regulatory mechanism of the circadian clock. This notion is supported by two lines of evidence. (1) The *Cab-1* transcript begins to accumulate toward the end of the dark period (Figs. 2A, 3A, and 4A). Because there is no change in the transcript stability, the accumulation of *Cab-1* mRNA most likely reflects an increase in transcription rate of the *Cab-1* gene in the dark. A similar increase in transcription rate in the dark is also seen with the *Cab*-CAT chimeric gene in transgenic tobacco (Fig. 4B). These results show that transcription of the *Cab* gene can occur without immediate light or phytochrome activation. (2) There is a precipitous drop in *Cab-1* transcript level shortly after midday (Figs. 2A, 3A, and 4A), which is most likely due to a drastic decrease in transcription rate in the light (Fig. 4B). This large drop in transcription rate occurs in spite of the presence of presumably saturating amounts of phytochrome. The above results are consistent with a model in which the *Cab-1* gene is regulated both positively by phytochrome and negatively by the circadian clock. The negative mode of regulation accounts for the transcriptional decrease in the light, observed with both the *Cab-1* and *Cab*-CAT chimeric mRNA in transgenic plants. This is substantiated further by the observation that repeated

red flashes throughout the night do not affect the dramatic drop in *Cab-1* RNA during the afternoon (data not shown). In this case, the inductive effect of phytochrome is overcome by the rhythm. The clock can thus be considered as a 'gate' that opens and closes rhythmically, allowing phytochrome to induce expression of the *Cab-1* gene only at a certain period during the morning.

What are the biological consequences for the plant in establishing such a rhythmic expression pattern for the *Cab* genes? In this context, it is interesting to note that the level of protein for the enzyme protochlorophyllide reductase, the light-dependent enzyme of chlorophyll biosynthesis, is regulated diurnally, being most highly expressed in the early morning (Griffiths et al. 1985). It therefore makes sense that the plant coordinates the expression of the major chlorophyll-binding protein with that of the key enzyme in chlorophyll biosynthesis. This may play a major role in the maintenance and assembly of the photosynthetic apparatus of the plant. Ultimately, plants possess rhythmic patterns that maintain an effective growth regime that is coordinated with the environment. The cyclic expression of the *Cab* genes may therefore be important in priming the light-harvesting capacity of the plant prior to the onset or the peaking of solar radiance.

Our studies also provide some considerations for differential mRNA stability. Although no cycling is observed in *Cab-1* mRNA abundance under control of the CaMV 35S promoter, the *Cab-1* mRNA is turning over quite rapidly. This is supported by the observation that in transgenic plants containing the whole *Cab-1* gene, the mRNA decreases to beyond detection within 4–6 hr during the afternoon. Interestingly, the CAT mRNA exhibits a much sharper 'spike' than does the *Cab-1* mRNA and falls off more rapidly, within 3 hr. This implies that this reported gene has an unstable message in transgenic tobacco, which has ramifications for its use in gene regulation studies.

We have shown recently that the phytochrome response of the *Cab-1* gene in etiolated transgenic tobacco seedlings is mediated by a 280-bp enhancerlike element (Nagy et al. 1987b). Whether the same element is involved in phytochrome response of the *Cab-1* gene in plants grown under L/D cycles remains to be elucidated. Further analyses of 5' deletion mutants and chimeric constructs will also allow us to delineate more precisely *cis*-acting elements that respond to circadian rhythm. We believe that identification of circadian-responsive *cis*-acting elements and *trans*-acting factors mediating this complex regulation will be a first step toward understanding the molecular basis of a large number of important biological processes, ranging from leaf movement to flowering.

Methods

Plant materials and light treatments

Wheat seeds (*Triticum aestivum* cv. *Era*) were imbibed in water overnight and then germinated and grown for 7 days on moist vermiculite in a growth chamber. For most experiments, plants were grown under a L/D cycle at a constant temperature of

24°C, with a light intensity of 1000 $\mu\text{mole/m}^2/\text{sec}$. This 7-day growth period preceded all treatments such as extended dark or light periods. However, for the constant illumination experiment, plants were both germinated and grown for 7 days in continuous white light (1000 $\mu\text{mole/m}^2/\text{sec}$) at 24°C. F1 seeds of transgenic tobacco plants were germinated on MS medium (Murashige and Skoog 1962). After rooting, the seedlings were transferred to soil and screened by S₁ analysis for expression of the *trans* genes. One plant was then chosen and vegetatively subcloned to provide a large number of identical plants. These subclones were placed in a growth chamber under L/D cycles for 3–4 weeks. For the measurement of RNA levels during dark periods, plants were placed in a black plexiglass cabinet within a darkroom maintained at 24°C. All manipulations in the darkroom were carried out under a dim green safelight. Extended white light illumination periods were performed in a growth chamber. For studies on the effect of phytochrome, plants were given either 30 min of red (660 nm), 30 min of far red (730 nm), or 30 min of red followed by 30 min of far red light. These red/far red treatments were administered at the end of the day after which the plants were immediately placed in the darkroom. Light sources and filters were as described (Nagy et al. 1987a). Wheat plants were harvested by cutting the uppermost 10 cm of the leaves. Transgenic tobacco tissue was harvested by taking only the third leaf pair of identical plants to avoid any artifacts produced by using leaves of different ages. All tissues were immediately frozen in liquid nitrogen upon harvesting.

Chimeric constructs and Ti-mediated gene transfer

The –1.8-kb *Cab-1*–CAT–E9–3' fusion, the *Cab-1* gene extending from –1.8 kb to +1.1 kb, as well as the –124 *Cab-1*–35S construct (Fig. 4) were cloned in the binary vector pMON505 and transferred to tobacco (Nagy et al. 1987a). F1 seeds from selfed plants were grown as described above.

Isolation and analysis of RNA

Total leaf RNA was isolated using aurin tricarboxylate as an RNase inhibitor (Nagy et al. 1987a). Slot blot analysis was performed using the Schleicher and Schull 'Minifold' apparatus according to the instructions of the manufacturer. Hybridization conditions were as described (Nagy et al. 1987a), using either a *Cab-1*-coding sequence probe (Nagy et al. 1986) or a wheat *rbcS* cDNA probe (Broglie et al. 1983). The analysis of the *Cab-1* RNA level was performed by 5' nuclease S1 protection assays using a single-stranded DNA probe derived from a pEMBL template (Nagy et al. 1987b). *Cab-1*–CAT–E9–3' RNA was analyzed by 3' nuclease S1 protection assays, using an end-labeled DNA probe as described (Kuhlemeier et al. 1987b). In equivalent experiments, RNAs were isolated on the same day (up to 120/day) and analyzed with the same probe preparation. Gels were run simultaneously, dried, and autoradiographed for identical times (normally 8 hr for 5' assays and 3 days for 3' assays).

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