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Expression of tobacco genes for light-harvesting chlorophyll a/b binding proteins of photosystem II is controlled by two circadian oscillators in a developmentally regulated fashion

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ABSTRACT **Light-induced expression of genes encoding the light-harvesting chlorophyll a/b binding proteins of photosystem II (Cab) was shown to be controlled by a circadian oscillator coupled to the red-light-absorbing plant photoreceptor phytochrome. Here we show that a red-light-insensitive oscillator is also involved in regulating the expression of the Cab genes. We provide evidence that germination leads, in a light-independent manner, to the setting and/or synchronization of endogenous oscillators and that it induces the expression of Cab genes in a circadian fashion. This circadian oscillator is not coupled to phytochrome, as it cannot be reset by red light for at least 44 h after sowing. Short red light pulses given between 12 and 44 h after sowing, however, induce new rhythms without perturbing the already free-running red-light-independent circadian oscillation. At this stage of development, the phytochrome-coupled and uncoupled circadian rhythms coexist. Both circadian rhythms are expressed and exhibit period lengths close to 24 h but are phased differently. At later stages of development (60 h or later after sowing), red light treatments synchronized these free-running rhythms and led to the appearance of a single new circadian oscillation. These data indicate that during early development the expression of single tobacco Cab genes, particularly expression of the *Cab21* and *Cab40* genes, is controlled in a developmentally dependent manner by two circadian oscillators.**

Biochemical and physiological processes in many organisms including prokaryotic cyanobacteria (1), fungi (2), the fruit fly *Drosophila melanogaster* (3), higher plants (4), and vertebrates (5) exhibit endogenous rhythms. Most of these rhythms show a periodicity close to 24 h (circadian) and are thought to be regulated by one or more internal biological oscillators (circadian clocks). Circadian regulation is a widespread phenomenon in plants, and the circadian oscillator(s) control plant functions ranging from stem elongation (6) and the movement of petals (7) to the rhythmic expression of several nuclear genes (for a review, see ref. 8). Our knowledge about the molecular mechanism by which the endogenous oscillator(s) modulates plant gene expression (output signal) or responds to changes in the environment (input signal) is rapidly increasing. The principal input stimulus (resetting signal) for most circadian oscillators characterized in plants is light, a signal that ensures synchronization of the endogenous oscillator to the natural day/night cycle. To sense the ever changing light environment, higher plants have developed several photoreceptors; among them the best characterized is the red/far-red light-absorbing phytochrome (for review, see ref. 9). Activation of phytochrome modulates the expression of several plant genes and these molecular events will ultimately lead to the initiation of photomorphogenesis (10, 11). Recent data show, however, that phytochrome has a dual role during plant development. Accumulating evidence indicates that in etiolated seedlings, activation of phytochrome, apart from modulating the expression of several plant genes, will also result in the setting or synchronization of the endogenous oscillator(s) (12–14). The phytochrome- and circadian-clock-modulated expression of various Cab genes (encoding the chlorophyll a/b binding proteins of the light-harvesting complexes of photosystems I and II) has been much studied (15, 16). Recent results clearly suggested that (*i*) phytochrome and the circadian clock control the expression of the Cab genes in seedlings and mature plants, at least partially at the level of transcription (17–19), and that (*ii*) the expression of the Cab genes and the setting or synchronization of the circadian oscillator(s) is regulated by the very low fluence response of phytochrome in etiolated wheat seedlings (20). In etiolated *Arabidopsis thaliana* seedlings, the phytochrome-regulated expression of Cab genes is preceded by a period during which their expression is controlled by a light-independent developmental program (21). Furthermore, Millar *et al.* (14) demonstrated that expression of the *A. thaliana Cab2–Luc* chimeric gene exhibits a weak but reproducible circadian oscillation in dark-grown transgenic *Nicotinana tabacum* seedlings.

We are interested in separating the phytochrome-mediated light-dependent regulation (resetting or synchronization) of the circadian clock from that of the transcription of Cab genes. These latter findings made it particularly interesting to characterize the interaction of phytochrome and the circadian clock(s) at different stages of seedling development. Therefore, we established an experimental system using tobacco seedlings grown under different light conditions. We determined the effect of light in inducing the circadian-clock-regulated expression of the Cab genes, particularly that of the *Cab21* and *Cab40* genes, during the first week of germination.

We report here that (*i*) the expression of the tobacco Cab genes is modulated by two circadian rhythms and that (*ii*) the induction of these circadian oscillations is developmental-stage-dependent.

MATERIALS AND METHODS

Plant Material. *N. tabacum* SR1 seeds were germinated in boxes (8 cm × 8 cm × 5.5 cm) that contained three layers of wet filter paper immersed in 3.5 ml of Hoagland medium (32). Boxes containing the seedlings were kept in growth chambers where the temperature (24°C) and humidity were constant. Seeds were germinated and grown (*i*) under light/dark cycles (16-h white light/8-h darkness; $I = 20$ W/m^2), (*ii*) in constant white light ($I = 20$ W/m^2, provided by Osram HQ IL bulbs), or (*iii*) in constant darkness. Alternatively, etiolated seedlings were irradiated with monochromatic red or far-red light. Light sources used in different experiments were as follows. Red

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Abbreviations: Cab, light-harvesting chlorophyll a/b binding proteins of photosystem II; CT, circadian time; LHC, light-harvesting complex.

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light: L_{max}, 658 nm; I, 6.8 W/m^2; half-bandwidth, 15 nm. Far-red light: L_{max}, 730 nm; I, 3.5 W/m^2. To allow comparison between different experiments, seeds were always sown at the hours of 0800 or at 2000. Samples (whole seedlings) were collected at 4-h intervals, for at least a 48-h period. Harvested plant material was immediately frozen in liquid nitrogen and stored at −80°C. Experiments described above were repeated at least three times. Results obtained by analyzing tissues derived from these experiments were always very similar and showed no significant alterations.

RNA Isolation and Northern Blot Experiments. Total RNA was isolated as reported by Nagy *et al.* (22). Transcript level of the whole tobacco Cab gene family or that of the *Cab21* (GenBank accession no. X52743) and *Cab40* (GenBank accession no. X52744) genes was determined by Northern blot hybridization experiments as described by Nagy *et al.* (22) with the following modifications. The expression level of the Cab gene family was monitored by hybridizing the filters with the [α-^{32}P]dATP-labeled fragment of the tomato light-harvesting complex (LHC) II type I *Cab1* gene. Hybridization and washing of the filters were performed under stringent conditions, as described (20). The expression levels of the tobacco *Cab21* and *Cab40* genes were determined by hybridizing the filters with [γ-^{32}P]dATP-labeled gene-specific (37 bp or 40 bp, respectively) single-stranded oligonucleotides probes. In these cases, filters were prehybridized for 16 h at 55°C in 6× SSC/5× Denhardt's solution/0.25% SDS/denatured salmon sperm DNA (100 μg/ml) and hybridized for 16 h at 55°C in the same solution containing probes at 3×10^7 cpm. Filters were then washed for 1 h at room temperature in 2× SSC/0.1% SDS and subsequently for two 15-min periods at 55°C in 1× SSC/0.1% SDS. Results of Northern blot hybridization experiments were then quantified. We have used rRNA as control RNA for rationing prior to subtraction. Quantification and subtraction of data were carried out by using a PhosphorImager (type F425: software, IMAGE QUANT version 3.3) manufactured by Molecular Dynamics.

RESULTS

Induction of Rhythmic Cab mRNA Accumulation in Developing Seedlings. The expression level and pattern of tobacco LHCII type I and II Cab genes, the *Cab21* (LHCII type I) and the *Cab40* (LHCII type I), were monitored in seedlings grown under various light conditions. Cab mRNA levels were determined by Northern blot hybridization experiments. We found that in 6- and 7-day-old seedlings, in all growth conditions tested, the Cab mRNA levels exhibited typical circadian rhythms (≈24 h long). These rhythms contained well-defined maxima and minima during this period (Fig. 1*A–D*). However, despite this similarity the Cab mRNA levels and the timing for induction of the circadian rhythms (the appearance of the first maximum and minimum) differed subtly, yet characteristically, in seedlings grown under different light/dark conditions. Fig. 1*A* shows that in seedlings grown under 16-h light/8-h dark cycles, Cab mRNA accumulation reached a maximum level on day 6 after germination and decreased afterward. The maxima were always at circadian time (CT) 8 (h) (CT0 = time of sowing) and the minima at CT20. We note that in these seedlings, the expression of the Cab genes reached detectable levels and showed the first circadian cycle on day 4 after germination (data not shown). In contrast, in seedlings grown in constant light, Cab mRNA levels increased continuously until day 5, reached detectable levels on day 4 (data not shown), and exhibited the first circadian cycle on day 6. From this time onward, although the level of Cab mRNA decreased dramatically, the expression of the Cab genes was clearly regulated by a circadian rhythm (maxima at CT0 and minima at CT16) (Fig. 1*B*).

The kinetics of Cab mRNA accumulation were quite different in dark-grown seedlings and in red-light-irradiated seedlings. Cab mRNA levels were too low to be detected until day 5 in red-light-irradiated dark-grown seedlings (Fig. 1*C*) and until day 6 in far-red-light-treated seedlings (data not shown) or dark-grown seedlings (Fig. 1*D*). However, they were clearly modulated by a circadian oscillator (maxima at CT0 and minima at CT12) from day 6 onward (Fig. 1*C*). In red-light-irradiated seedlings, the maximum level of Cab mRNA accumulation was ≈4-fold lower than the level in seedlings grown in 16-h light/8-h dark cycles. The maximum level of Cab gene expression was significantly lower (≈10-fold) in far-red-light-treated seedlings (data not shown) or in seedlings grown in complete darkness (15- to 20-fold lower) (Fig. 1*D*). In these seedlings, even at these very low expression levels, the accumulation of Cab mRNA showed a well-defined maximum (CT0) and minimum (CT12) on day 7 and was regulated by a circadian oscillator (Fig. 1*D*). Furthermore, Fig. 1*E* shows that the steady-state mRNA level of the tobacco *PhyA1* gene (23), in contrast to the cycling Cab mRNA, is not regulated by a circadian oscillator.

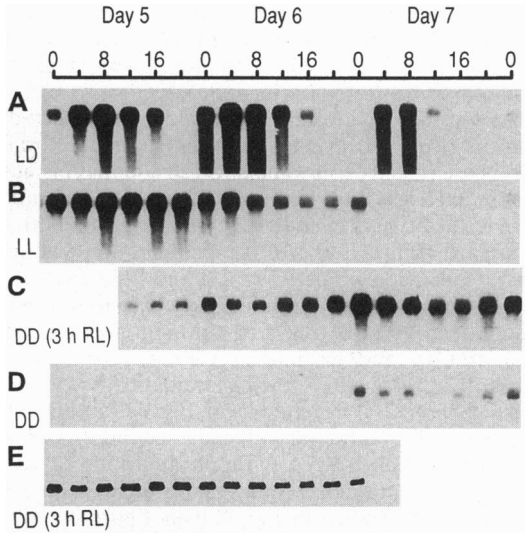

FIG. 1. Circadian clock regulates the accumulation of Cab mRNA (*A–D*) but not that of the PhyA mRNA (*E*) in 5- to 7-day-old tobacco seedlings. Seedlings were grown under 16-h light/8-h dark cycles (LD) (*A*), grown in constant light (LL) (*B*), irradiated for 3 h with red light [DD (3 h RL)] immediately after sowing but returned to constant darkness for growth (*C* and *E*), and grown in constant darkness (DD) (*D*). Numbers indicate the CT based on a value of ≈24 h for τ (period length of the circadian rhythm). Seeds were uniformly sown at the hour of 0800 (CT0). Each lane contains 10 μg of total RNA (*A* and *B*) or 20 μg of total RNA (*C–E*). Filters were exposed for 48 h (*A* and *B*) or for 96 h (*C–E*).

Fig. 2 shows that the induction of rhythmic accumulation of

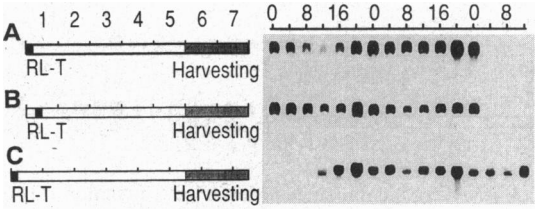

FIG. 2. Sowing and/or germination induces rhythmic Cab mRNA accumulation in a red-light-independent fashion. Seeds were sown at 0800 (*A* and *B*) or at 2000 (*C*). Seeds were treated with red light for 3 h after sowing (*A* and *C*) or were germinated for 9 h in dark, treated with red light for 3 h, and returned to constant darkness (*B*). RL-T, red light treatment. (*Left*) The numbers 1–7 indicate days after sowing. (*Right*) Numbers show the CT (as in Fig. 1) on the days of harvesting. Harvesting started at CT0 (*A* and *B*) or at CT12 (*C*). Each lane contains 20 μg of total RNA. Each filter was uniformly exposed for 48 h.

Cab mRNA in germinating seeds is indeed a light-independent process. Irrespective of red-light treatments given either at the time of sowing (Fig. 2*A* and *C*) or 9 h after sowing (Fig. 2*B*), the accumulation of Cab mRNA in each experiment (*i*) reached approximately the same level, (*ii*) was clearly regulated by a circadian clock, and (*iii*) showed free-running rhythms exhibiting the same phase on day 7 (maxima at CT20 and minima at CT8).

Light-Induced Rhythmic Accumulation of the Cab mRNA Is Developmental-Stage-Dependent. The developmental stages at which the rhythmic Cab mRNA accumulation was induced by light were also determined. Fig. 3*A* and *B* shows once more that red light pulses given within the first 12 h after sowing did not change the phase of the free-running circadian rhythm (maxima at CT4 and minima at CT12). Red light pulses applied 36 h or 44 h after sowing dramatically changed the patterns of Cab mRNA accumulation. Fig. 3 *C* and *D* indicates that these red light treatments induced a second rhythm for the accumulation of Cab mRNA without abolishing or resetting the existing free-running rhythm. Quantitative analysis of the complex pattern is shown in Fig. 4. This figure clearly demonstrates that subtraction of the red-light-insensitive rhythm from those obtained after the second red-light treatments resulted in the appearance of new circadian rhythms. These new circadian rhythms, induced by red light pulses at 36 h (Fig. 4*C*) and at 44 h (Fig. 4*D*), apparently have their maxima (CT16–20) and minima (CT4) at similar circadian times. Therefore, we concluded that these latter circadian rhythms, measured at the level of Cab mRNA accumulation, coexist with the light-insensitive circadian rhythm. These rhythms are not coincident and each has periodicity close to 24 h. In contrast, red light irradiation at or after 60 h always led to the induction of a single circadian rhythm, regardless of earlier light treatments (Fig. 3*E*).

We used the same RNA preparations and repeated the above described experiments using gene-specific chemically synthesized oligonucleotides as probes. We found that the mRNA accumulation of the tobacco *Cab21* gene and of the *Cab40* gene produced very similar circadian patterns. For example, Fig. 5 clearly shows that the expression of these Cab genes was also modulated by two circadian rhythms after a second red light treatment (36 h after sowing). These coexisting but differently phased circadian rhythms were stable and readily detectable on days 8 and 9 after sowing (data not shown).

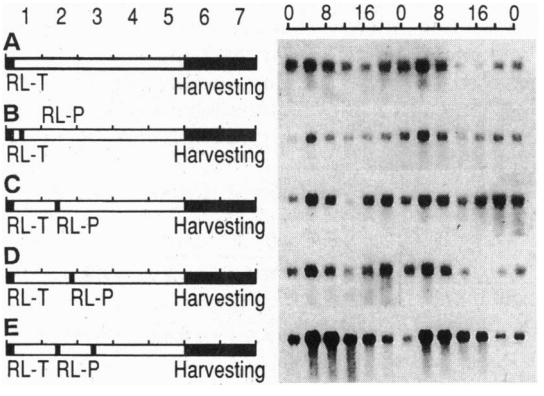

FIG. 3. Developmental stage-specific light induction of rhythmic Cab mRNA accumulation in tobacco seedlings. Seeds were uniformly sown at 0800 (CT0), irradiated for 3 h with red light (CT0–3), and returned to darkness. Germinating seeds were then, with the exception of control seeds (*A*), illuminated again with a short (5 min) red light pulse at 12 h (*B*), 36 h (*C*), 44 h (*D*), or 36 h and 60 h (*E*) after sowing and returned to constant darkness. RL-T, 3-h red light treatment; RL-P, 5-min red light pulse. (*Left*) Numbers indicate the days after sowing. (*Right*) Numbers show the CT at the days of harvesting. Each lane contains 20 μg of total RNA. Filters were uniformly exposed for 48 h.

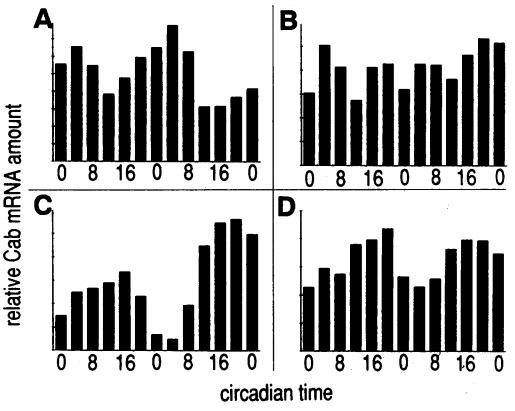

FIG. 4. Accumulation of tobacco Cab mRNA is modulated by two circadian rhythms. Data of Northern blot hybridization experiments (shown in Fig. 3) were quantified by PhosphorImager (software, IMAGE QUANT version 3.3). *A* corresponds to Fig. 3*A* and *B* corresponds to Fig. 3*C*. (*C* and *D*) The newly induced circadian rhythms were obtained by subtracting *A* from *B* or *A* from Fig. 3*D*, respectively. The quantification of Fig. 3*D* is not shown.

DISCUSSION

Light-Independent Induction of Rhythmic Cab mRNA Accumulation in Germinating Seeds. Recent reports have clearly established that the short red light treatments of etiolated seedlings a few days old induced the transcription and rhythmic accumulation of Cab mRNA in various monocot and dicot species (12–14, 20). In this paper, we provide evidence that the setting or the synchronization of the circadian oscillator at the very early stage of development occurs in a light (phytochrome)-independent fashion. We showed that Cab mRNA accumulation exhibited identical circadian oscillation (minima and maxima occurred at the same CT) in seedlings (*i*) grown in constant darkness (Fig. 1*D*), (*ii*) grown in constant light (Fig. 1*B*), (*iii*) irradiated with red light [at various times but not later than 12 h after sowing (Figs. 1*C* and 2*A–C*)], or (*iv*) even irradiated with continuous red or far-red light (data not shown). We showed clearly that the time of sowing (Fig. 2*C*), rather than other factors such as light treatments (Fig. 2*A* and *B*) or growth conditions, is important to define the phase of the endogenous circadian rhythms. Notwithstanding the similar circadian rhythms, the Cab transcripts accumulated to different levels. The highest levels were in light-grown (Fig. 1*A* and *B*) and red-light-pretreated (Fig. 1*C*) seedlings, lower levels were in far-red-light-illuminated seedlings (data not shown), and lowest levels were in dark-grown seedlings (Fig. 1*D*). Therefore, we suggest that at this early developmental stage (*i*) phytochrome may mediate the light-induced transcription of Cab genes but (*ii*) it is not involved in setting or synchronizing the circadian clock(s). Furthermore, these data indicate that the setting or synchronization of the circadian clock(s) did not occur during seed development, and thus, the circadian clock is probably induced by the early molecular events of seed germination. These data corroborate and extend results obtained by Millar *et al.* (14) and Nagy *et al.* (19). These authors showed that the expression of the wheat *Cab1* gene in seedlings grown in constant light (19) or expression of the *A. thaliana Cab2* gene in transgenic tobacco seedlings grown in constant darkness (14) is regulated by a circadian clock. These previous observations and our present data suggest that, in germinating seeds, the setting or synchronization of the circadian oscillator(s) under constant environmental conditions (even in constant light) is uncoupled from the photoreceptor phytochrome. Phytochrome-uncoupled induction of the rhythmic accumulation of Cab mRNAs has recently been described by Kloppstech *et al.* (24). These authors demonstrated that in dark-grown bean seedlings cyclic heat-shock treatments could

also result in the induction of the rhythmic accumulation of Cab mRNAs. Brusslan and Tobin (21) reported that the light (phytochrome)-independent expression of the *Cab1* gene is not affected by the circadian clock in *A. thaliana* seedlings. These authors found that (in seedlings illuminated for 15 min with white light after sowing but grown in darkness) the accumulation of Cab1 mRNA increased steadily until day 4 and then decreased afterward. We assume that the apparent contradictions between conclusions drawn by Brusslan and Tobin (21) and those reported in this paper and by Millar *et al.* (14) could be due to (*i*) differences of molecular programs controlling the development of tobacco and *A. thaliana* seedlings and/or (*ii*) the different experimental protocols followed.

Phytochrome-Regulated and Phytochrome-Independent Circadian Rhythms Altering Cab Gene Transcript Levels Can Coexist in Developing Seedlings. We demonstrated that 5-min red-light pulses given 12, 36, or 44 h after imbibition will not abolish or reset the germination-induced free-running rhythm of Cab mRNA accumulation (Figs. 3 and 4). These treatments, however, led to the induction of new circadian rhythms 36 or 44 h after imbibition. Therefore, we concluded that (*i*) at this developmental stage, the rhythmic accumulation of Cab mRNA is affected by two circadian oscillations (a phytochrome-uncoupled and a phytochrome-coupled circadian oscillations) and that (*ii*) in the absence of further light pulses these circadian rhythms coexist but are phased differently. Hennessey and Field (25) provided evidence that multiple oscillators with different intrinsic frequencies control stomatal opening and photosynthesis in bean plants. More recently, Roennenberg and Morse (26) elegantly proved that multiple oscillators operate in the unicellular algae *Gonyaulux*. Independent of these results, the activity of multiple circadian oscillators has also been postulated based on the so-called "splitting" phenomenon. It has been reported that under constant conditions, the rhythm of locomotor activity in birds (27) or leaf movement in plants (28) may split into two components. These split rhythms then run for a while before they become resynchronized with each other at a new phase relationship. It was postulated that splitting is provoked by the temporal modification of coupling between independent oscillators and, therefore, it reflects the transient separation of these circadian oscillators (27). However, we demonstrated in our experiments that the coexisting but differently phased rhythms are stable and free-running, at least, for 100 h after the light treatments. Therefore, it is unlikely that this circadian oscillation would be transient and caused by splitting.

In addition, we provide evidence that two circadian rhythms regulate the expression of a single plant gene. Our data strongly suggest that mRNA accumulation of single tobacco genes, particularly from the *Cab21* but also from the *Cab40* gene (determined by gene-specific probes, Fig. 5), is controlled by two independent circadian oscillators.

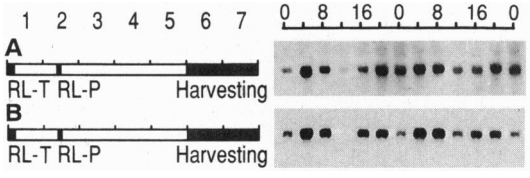

FIG. 5. Accumulation of the Cab21 and Cab40 mRNAs is affected by two circadian rhythms. Filters corresponding to Fig. 3*C* were hybridized with Nt-Cab-21 (*A*) or Nt-Cab-40 (*B*) gene-specific single-stranded oligonucleotides. Nt-Cab-21, 5′-GAAAAGAAAAGTGAGTGTTT-GATGGTTTGAAATGCCC-3′, Nt-Cab-40, 5′-TTTTTTTTATG-GCTGAAGTAATAGAGAAGTTAGCTGTGGT-3′. (*Right*) Each lane contains 20 μg of total RNA, and filters were uniformly exposed for 48 h. RL-T, 3-h red light treatment; RL-P, 5-min red-light pulse. Numbers indicate the CT. (*Left*) Numbers indicate the days after sowing.

Appearance of Circadian Rhythms Affecting the Expression of Cab Genes Is Developmentally Regulated. Our present and recent data (15, 19, 20) suggest that (*i*) during seedling development, Cab gene transcript levels are regulated by at least two circadian rhythms, (*ii*) these rhythms are induced at different stages of development, and (*iii*) the rhythms are differentially regulated by the photoreceptor phytochrome.

It has been shown recently that the phytochrome-coupled circadian clock regulates the expression of Cab genes at the level of transcription (18, 19). Cis-regulatory elements and transcription factors mediating clock-responsive gene expression were recently identified (17, 29). Members of the signal transduction pathway(s) required for phytochrome-regulated gene expression in higher plants (i.e., heterotrimeric G proteins, Ca^{2+}, and cGMP) have recently been identified (30, 31). The mode of regulation [i.e., how the circadian clock and phytochrome interact and at what level and how the clock-initiated pathway(s) interfere with the phytochrome-dependent pathway(s)] remain to be determined.

Independent of the type of regulation, our data define three stages of seedling development that may reflect the different levels of a complex developmental program. These stages can be defined as (*i*) 0–12 h after sowing, where induction of circadian-rhythm-modulating Cab gene mRNA levels is light independent; (*ii*) 12–44 h after sowing, where light (phytochrome)-dependent rhythms are induced but without phase shifting the free-running light-independent rhythm; and (*iii*) 60 h or later after sowing, where newly given light pulses will lead to the appearance of a single "unified" rhythm apparently by phase shifting all earlier induced rhythms.

Under all the conditions tested (except in seedlings grown in 16-h light/8-h dark cycles), Cab mRNA accumulation was first detectable on day 4 after sowing. Thus, the results of all light treatments given during the first 60 h after sowing were detectable only after day 4 of sowing. We could not determine the immediate effects of these light treatments on the synchronization or resetting of the circadian oscillator(s) [unlike the phase-shifting experiments that were performed at developmental stages 84 h after sowing or later (20)].

These observations provoke several interesting questions. (*i*) Are the regulatory mechanisms by which the phytochrome-coupled and uncoupled circadian oscillator(s) modulate Cab gene expression identical or different? (*ii*) What phytochrome species are involved in mediating the phytochrome-coupled circadian clock-controlled expression of the Cab genes at these different developmental stages? (*iii*) What happens to the phytochrome-uncoupled circadian oscillator at later stages of development (60 h after sowing or later)? Will it be coupled to phytochrome directly (by the emergence of a new pathway) or indirectly (by the phytochrome-coupled circadian oscillator)? Or does it no longer exist?

The first two questions can be answered by studying the expression of different chimeric Cab genes in transgenic plants (17, 18) or in different *A. thaliana* mutants lacking specific phytochrome species (11). For the third question, we believe that our data provide a reasonable background for designing specific screening methods for the isolation of at least two types of circadian clock mutants.

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