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The circadian oscillator is regulated by a very low fluence response of phytochrome in wheat

(chlorophyll a/b binding protein/circadian rhythm)

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ABSTRACT **Expression of genes encoding the light-harvesting chlorophyll a/b binding proteins of photosystem II (Cab) in etiolated wheat seedlings is controlled by phytochrome and a circadian clock. Even photoconversion of <1% of phytochrome to its active form, which can be achieved by moonlight, induces the expression of the Cab genes, particularly that of the Cab-1 gene, in circadian fashion. Thus, this reaction shows the characteristics of a low and a very low fluence response. A single far-red light pulse given to an etiolated seedling is sufficient for a persistence of the circadian oscillation of the Cab-1 mRNA level for at least 100 h. Subsequent red (R) or long-wavelength far-red (RG9) light irradiations alter the free running rhythm. These observations indicate a change in sensitivity to phytochrome and/or a control by stable phytochrome. The latter hypothesis is supported by the observation that the level of Cab-1 mRNA is increased or decreased by a second R or RG9 light pulse, respectively.**

Biochemical and physiological processes in many organisms are known to exhibit endogenous rhythms. Most of these rhythms show a period close to 24 h (circadian) and are thought to be regulated by an internal biological oscillator (circadian clock) (1). In nature, there is a constant interaction between the environment and the circadian clock. Changes in the environment (for example, light/dark transitions) modulate (reset) the activity of the endogenous oscillator and result in phase shifts of the free running rhythms (2, 3).

In plants a large number of activities are controlled by the cyclic alteration of day and night (4, 5). To sense the ambient light quality and quantity, plants evolved several photoreceptors; the best characterized among these is phytochrome (6). Phytochrome exists in two interconvertible forms. In dark it is synthesized as red-absorbing phytochrome (Pr), which is physiologically inactive; light absorption (L_{max} = 660 nm) converts Pr to far-red-absorbing phytochrome (Pfr), which is the biologically active form. Pfr is recycled to Pr by absorption of far-red light (L_{max} = 730 nm). Based on fluence response studies using monochromatic red light, a low fluence response is observed above 1% Pfr and a very low fluence response (VLFR) is obtained below this level. Because of the overlapping absorption spectra of Pr (L_{max} = 660 nm) and Pfr (L_{max} = 730 nm), saturating light pulses of red (R), far-red (FR), and long-wavelength far-red (RG9) light will establish photoequilibria of *ca.* 80%, 2–3%, and <0.1%, respectively (7). Therefore the LFR can be characterized by the classical reversion experiments using red (600 nm) and far-red (720 nm) light. The VLFR that can be induced by moonlight or even starlight is saturated by far-red (720 nm) light. Only long-wavelength far-red (RG9) light with a maximal emission at 800 nm can partially revert the induction of the VLFR (8, 9).

Several laboratories reported that the light-induced expression of a number of genes is mediated by phytochrome (for review, see refs. 10–12). More recently it became evident that the phytochrome-regulated expression of some of these genes is further modulated by an endogenous oscillator (13–15). Especially thorough studies have addressed the phytochrome- and circadian-clock-regulated expression of various Cab genes [encoding the chlorophyll a/b binding proteins of the light-harvesting complexes I and II (13, 16–18)]. Several interesting points have emerged from these studies. (*i*) Expression of the various Cab genes is regulated, at least partially, at the level of transcription (14, 17, 19). (*ii*) Since the expression of the Cab genes continues to oscillate in constant light, when the Pfr/Pr ratio is high, it is likely that the clock exerts a negative control over the gene activation by phytochrome (16). (*iii*) In constant darkness, the expression of the Cab genes can continue in a cyclic manner for at least 48 h, indicating the possible involvement of a long-lived Pfr pool in the activation process. (*iv*) In etiolated tissues a short R light pulse resulted in rhythmic accumulation of the translatable Cab mRNA, indicating the involvement of phytochrome in setting or synchronizing the clock (20).

This latter finding makes it particularly interesting to determine the Pfr requirements for inducing and/or rephasing the circadian-clock-controlled expression of the Cab genes. Therefore we established a 24-h phase–response curve for the wheat Cab-1 mRNA accumulation induced by FR light and we determined the effect of an interruption by R or RG9 light pulses.

We report here that, in wheat seedlings, the setting of the endogenous oscillator or the synchronization of the running rhythms is likely to be a VLFR. In addition, we show that a single light pulse will lead to a circadian oscillation of Cab mRNA over a period of 100 h. The long persistence of the rhythm can be explained either by an increased sensitivity to Pfr or by the control of a stable, long-lived Pfr pool.

MATERIAL AND METHODS

Plant Material. Wheat seeds (*Triticum aestivum* L. cv. Eve) were imbibed in water overnight and grown in complete darkness at constant temperature (24°C) for 3.5 days. Etiolated seedlings were irradiated for 5 min with R (L_{max} = 660 nm, 6.8 $W \cdot m^{-2}$), FR (L_{max} = 730 nm, 3.5 $W \cdot m^{-2}$), or RG9 (L_{max} = 750 nm, 7 $W \cdot m^{-2}$, with <0.1% transmission at wavelength shorter than 710 nm) light as described (21). These light sources establish the photostationary state of

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Abbreviations: Cab, chlorophyll a/b binding proteins of photosystem II; VLFR, very low fluence response; Pr, red-absorbing phytochrome; Pfr, far-red-absorbing phytochrome.

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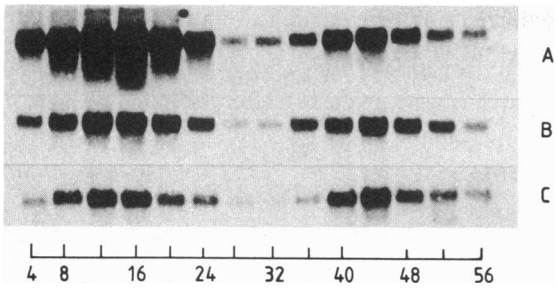

FIG. 1. Northern blot analysis of light-induced, rhythmic Cab mRNA accumulation. Etiolated wheat seedlings (3.5 days old) were irradiated with either 5-min R (*A*), FR (*B*), or RG9 (*C*) light and then returned to darkness. Samples were collected every 4 h. Each lane contains 20 μg of total RNA.

phytochrome within <5 min (22). For phase-shift experiments the seedlings were grown in darkness for 24–48 h after the first light treatment (FR) and were then irradiated a second time for 5 min with either R or RG9 light. Leaves were harvested at 4-h intervals for up to 100 h after the first light treatment. After harvesting, the samples were immediately frozen in liquid nitrogen and stored at −80°C until further processing.

RNA Isolation, Northern Blots, and S1 Nuclease Protection Experiments. Total RNA was isolated as described (23). The transcript level of the wheat Cab genes was determined by Northern hybridization experiments using 20 μg of total RNA in each sample. Northern hybridizations were also performed as described (23). A *Bst*XI–*Sma* I fragment containing the full coding region of the Cab-1 gene (24) was isolated, labeled by random priming, and used as probe. The transcript level of the Cab-1 gene was measured by 5′ S1 nuclease protection assays. Total RNA (15 μg) was hybridized to a single-stranded Cab-1 gene-specific probe. The preparation of the single-stranded, labeled probe and further manipulations (hybridization, S1 nuclease reaction, and electrophoresis) were as recently described (25). The protected fragments were visualized by autoradiography. S1 nuclease protection assay data were quantitated by measuring the absorption of bands with a laser densitometer (2202 UltraScan, LKB). The measured absorption values were then translated to digital data by an integrator (3390 A, Hewlett–Packard). The experiments were repeated in three independent series and showed qualitatively the same results with slight fluctuations in peak positions and amplitudes. A representative set of data (Figs. 1–5) obtained in one independent experiment is presented.

RESULTS

Photoinduction of Rhythmic Cab mRNA Accumulation. The induction of Cab mRNA accumulation, after a single light pulse, was analyzed in etiolated wheat seedlings. mRNA levels for the whole Cab gene family were determined by Northern blot experiments (Fig. 1). The Cab-1 gene-specific transcript levels were monitored by S1 nuclease protection assays (Fig. 2). We found that R, FR, and RG9 light pulses effectively induced a rhythmic Cab mRNA accumulation. The initial maxima appeared at 12 h and the second maxima appeared at 44 h after the light pulse. Northern blot analysis showed a rapid dampening of the second maxima after a R light pulse (Fig. 1*A*), a constant level after RG9 (Fig. 1*C*), and a slight decrease after FR light illumination (Fig. 1*B*). The expression of the Cab-1 gene showed a similar pattern but with an indication of higher levels of Cab-1 mRNA in the second maximum after RG9 irradiation (Fig. 2*C*) and slightly stronger increase after FR light pulse (Fig. 2*B*; Fig. 3, A).

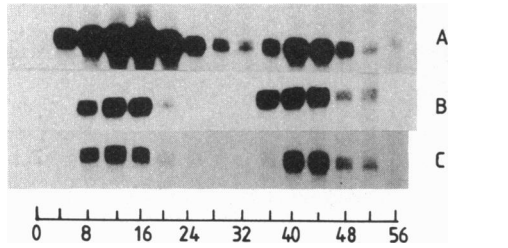

FIG. 2. Analysis of the light-induced, rhythmic Cab-1 mRNA accumulation. Aliquots of total RNAs used in Northern blot experiments were also analyzed by S1 nuclease protection assays. Each lane contains 15 μg of total RNA. (*A*) R light. (*B*) FR light. (*C*) RG9 light.

Phase–Response Curves for Photoinduction of Cab-1 Accumulation. The Cab-1 mRNA accumulation was analyzed up to 100 h after a single FR light pulse (Fig. 3, A). Maxima were

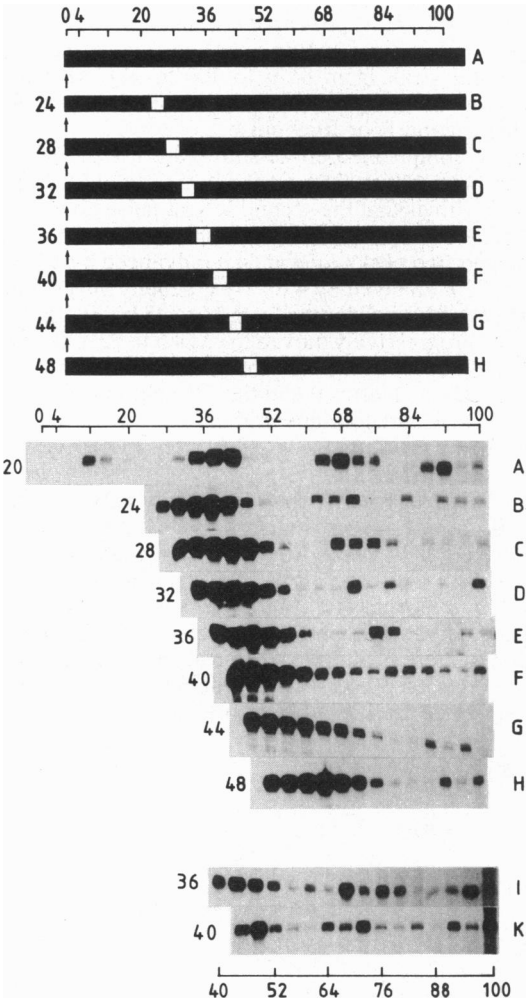

FIG. 3. Modulation of the phase of Cab-1 gene expression by a second light pulse. (*Upper*) Experimental design. (*Lower*) Actual data of one experiment. (*Upper*) Etiolated wheat seedlings (3.5 days old) were irradiated with a 5-min FR light pulse (arrows) and then returned to darkness (solid bars). The seedlings either received no further light treatment or were exposed to a 5-min R or RG9 light pulse (open squares) at various times after the first FR light pulse. A, no additional light treatment after the initial FR pulse; B–H, 5-min R light treatment; I and K, 5-min RG9 light treatment. Samples were collected every 4 h up to 100 h after the first FR pulse. (*Lower*) The amount of Cab-1 mRNA was assayed by 5′ S1 nuclease protection. Each lane contains 15 μg of total RNA. Samples, except those shown in I and K, were exposed uniformly for 16 h. Samples for panels I and K were exposed for 32 h.

obtained at 12, 44, 68, and 92 h after the light pulse. To test for the possibility of inducing a phase shift, a second light pulse was given at 24, 28, 32, 36, 40, 44, and 48 h after the first pulse. The second light pulse was either R or RG9 light. A detailed description of the phase-shift experiments is given in Fig. 3 *Upper*. We found that the second R light pulse was always inductive—i.e., it resulted in Cab-1 mRNA maxima higher than the one measured after the first FR pulse (Fig. 3, B–H). By contrast, the second RG9 light pulse consistently led to an initial reduction of the Cab-1 mRNA accumulation. Data for the second RG9 light pulses are shown only for irradiations 36 and 40 h after the inductive FR pulse (Fig. 3, I and K). Compared to that of the control (Fig. 3, A), the positions of the first (44 h) maxima were promoted for irradiations with R or RG9 light at 24–32 h and progressively delayed by irradiations at 36–48 h. Fig. 4*A* shows the phase–response curve for the 44 h (first) peak.

Irradiations 24 h after the first FR light pulse with R (Fig. 3, B) or RG9 light (data not shown) resulted in a shift of the original fourth (92 h) peak to 84 h. Varying the second R or RG9 light pulses from 24 h up to 40 h resulted in a progressive shift of this peak from 84 h to 100 h. Fig. 4*B* shows the phase–response curve for the fourth (92 h) peak obtained after the second R or RG9 pulse.

A more complicated phase-shift pattern was observed for the third peak that appears 68 h after the initial FR light pulse. Fig. 3, B, shows that the second R light pulse given 24 h after the first FR light pulse produced a split in the 68-h maximum, resulting in two peaks, one at 64 h (advanced by 4 h) and the other at 72 h (delayed by 4 h). By gradually varying the time for the second R irradiation from 24 to 48 h, the "advanced" peak was progressively moved from 64 h to 92 h. By contrast, the "delayed" peak moved from 72 h to 76 h and then to 80 h (irradiation 32 h after the initial FR light pulse). This shift, however, does not continue: rather, a new peak at 60–68 h is observed (irradiations at 36–48 h after the initial FR light pulse).

If RG9 light is used for the second light pulse, again a split of the 68-h peak occurs (Fig. 3, I and K). As the time of the second RG9 light pulse was gradually changed, both new peaks at 64 h (advanced) and 72 h (delayed) shifted to 100 h and 92 h, respectively (Fig. 4 *B* and *C*). For each 4-h movement of the light pulse, both peaks are shifted by 4 h. In addition to these peaks, irradiation at 28 h or later resulted in a new series of peaks, starting at 60 h and progressively working up to 84 h. Furthermore, a new series of peaks started at 60 h and moved up to 76 h for irradiations at 36 h or later. A quantitated Cab-1 mRNA accumulation pattern for the basic FR light and for a second R or RG9 light pulse (in this case they were given 36 h after the initial FR light pulse) is shown in Fig. 5.

DISCUSSION

Oscillating Cab-1 mRNA Accumulation Is Induced by VLFR. The induction of the Cab transcription in grass (barley and wheat) seedlings is extremely sensitive to light (26) and belongs to the class of VLFRs that is generally believed to be mediated by labile phytochrome (27). We show here that the rhythm of Cab-1 mRNA accumulation in 3.5-day-old, dark-grown wheat seedlings also can be induced by R, FR, and RG9 light, establishing, in this sequence, 80%, 2%, and <0.1% Pfr (7). This indicates that not only the transcription of Cab genes but also the circadian rhythm is induced at a very low level of Pfr. Due to the very low transcript level we were not able to establish an expression pattern for the Cab-1 gene in dark-grown, etiolated seedlings. Consequently, our data can be explained either by synchronization of running rhythms or by induction of a new rhythm after resetting the circadian oscillator.

Is a Second Light Pulse Able To Induce a Phase Shift? For etiolated bean seedlings Tavladoraki *et al.* (20) reported that a second R pulse after the first inductive R light pulse led to a complete phase shift measured at the level of translatable Cab mRNA. This shift occurs when the second light pulse was given at the trough but not at the peak. A FR light pulse did not initiate the fluctuating expression of the Cab genes or reset the clock but did lead to a decrease of the Cab gene expression level.

The apparent disagreement with our data shown here probably derives from the fact that in bean seedlings the expression of the Cab genes may be less sensitive to low levels of Pfr than in wheat. In the case of wheat seedlings, R light pulses given between 24 and 48 h after the first inductive FR pulse always led to a strong induction of Cab-1 mRNA accumulation (Fig. 3). This indicates that, during the full cycle of the rhythm, the system is sensitive to Pfr formed by the second light pulse (27).

The second R and RG9 light pulses, apart from affecting the expression level of the Cab-1 gene, also resulted in a clear alteration of the second and third peaks of the Cab-1 mRNA accumulation pattern, which can be interpreted as a phase

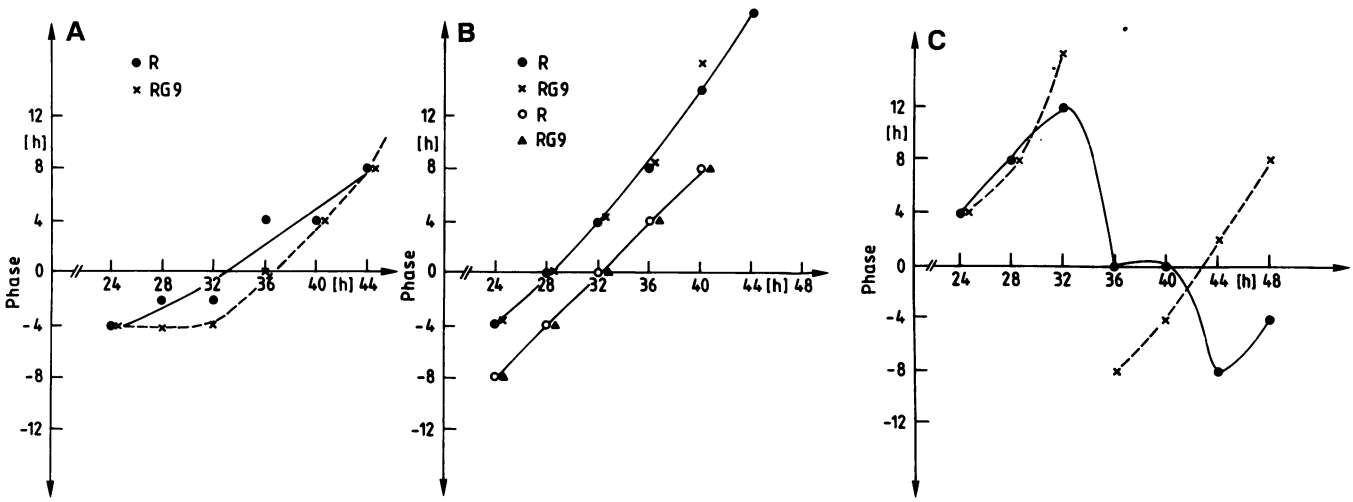

FIG. 4. Phase–response curve for the effect of a second 5-min R or RG9 light pulse on rhythmic Cab-1 mRNA accumulation after an inductive 5-min FR light pulse. (*A*) Phase–response curve for the peak at 44 h. ●, R light; ×, RG9 light. (*B*) Phase–response curves for the delayed curves of the peak at 68 h (●, R light; ×, RG9 light) and at 92 h (○, R light; ▲, RG9 light). (*C*) Phase–response curve for the advanced curves of the peak at 68 h (●, R light; ×, RG9 light).

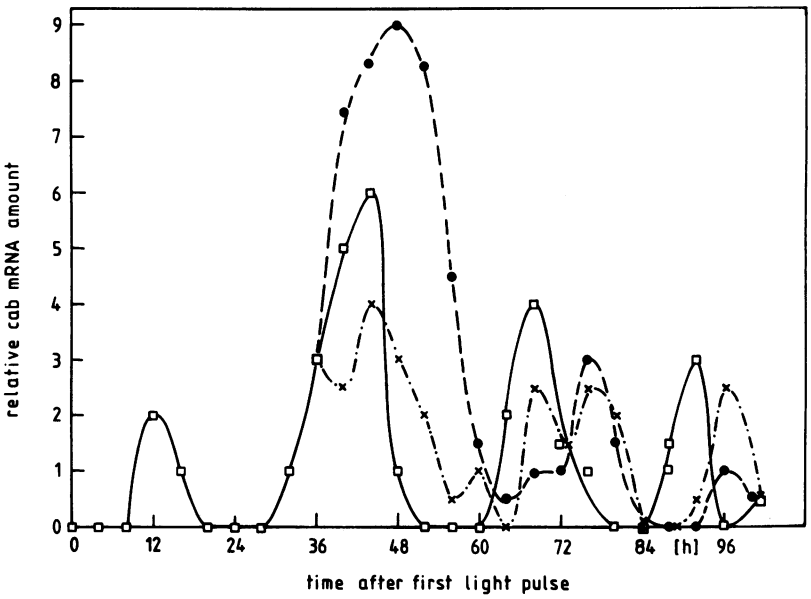

FIG. 5. Quantitation of Cab-1 mRNA accumulation after a single 5-min Fr light pulse (□) and after a second 5-min R (●) or RG9 (×) light pulse given 36 h after the first FR light pulse.

shift. The phase–response curves (Fig. 4) show that pulses given at the trough between the first and second peaks (i.e., 24–48 h) resulted in advanced second, third, and fourth peaks. Irradiation at the peak position resulted in a slight delay of the second peak. The analysis of the third and fourth peaks is complicated by the fact that the second irradiation resulted not only in a phase shift but also in a splitting of the third and fourth peaks into advanced and delayed peaks. Because of the differences in the phase–response curves for the second and third peaks, the delay between these peaks increased from 24 h to 36–40 h for the FR control curve. This is compensated for by the appearance of new peaks. The observed complex patterns of Cab-1 mRNA accumulation indicate that short, monochromatic light pulses (R and RG9) applied at a certain time after the initial pulse will disrupt the subsequent peaks rather than totally reset the clock!

Is the Accumulation of the Cab-1 mRNA During the Rhythm Always Dependent on the Presence of Pfr? The rhythmic accumulation of Cab-1 mRNA is maintained even after a single FR light pulse for at least four cycles (100 h, Fig. 3, A). The second (44 h) maximum of the accumulation curve is higher (Fig. 3, A) or at least the same as the first (12 h) maximum after the initial FR pulse (Fig. 1*B*). In addition we found that the distance between the first and second peaks (32 h) is atypical for a circadian rhythm (Figs. 1–3). These results give rise to the following question: Is the sensitivity of the signal transduction chain, controlling the Cab gene expression, constant to Pfr during the prolonged dark period?

The observation that the Cab-1 mRNA accumulation is affected by a second R and RG9 light pulse (Fig. 3, A–K) indicates that it can respond to changes in Pfr levels. Since it is not expected that Pfr levels will increase in the dark, the observation that the second peak has higher mRNA levels than the first indicates that the sensitivity to remaining Pfr should increase. Consequently, even the prolonged expression after a single FR light pulse could be explained by changes in sensitivity to Pfr.

The appearance of an atypical distance between the first and second peak is not unique to this system. Wehmeyer *et al.* (21) reported similar findings by studying the expression of tobacco Cab genes in etiolated seedlings. It is interesting to note, in this context, that developmental control of sensitivity to Pfr for Cab gene regulation has recently been reported (28). Therefore it is conceivable, based on this latter finding, that the appearance of non-circadian distance between the first two maxima may be related to changes in sensitivity to Pfr.

Does Stable Phytochrome Control the Cab-1 mRNA Accumulation? A RG9 light pulse given between 24 h and 48 h after the first inductive FR light pulse consistently led to an initial reduction of the Cab-1 mRNA accumulation (Fig. 3, I and K; Fig. 5). This indicates that Pfr must be present 24–48 h after the inductive FR light pulse to allow high levels of Cab-1 mRNA accumulation during the second peak of the rhythm. The reduction of the Cab-1 mRNA level by RG9 light thus shows that the RG9 light will decrease the remaining level of Pfr. Consequently, we assume that stable Pfr is necessary for the sustained rhythmic accumulation of the Cab-1 mRNA.

Taken together, we can interpret our data such that labile and stable phytochromes control the expression of the Cab-1 gene and that, in addition, the sensitivity to Pfr will change during development of the seedling. These assumptions can be tested by studying the photoregulation of Cab gene expression either in mutants diminished specifically in labile and stable phytochromes—i.e., hy-1, hy-2, and hy3 mutants of *Arabidopsis thaliana* (29)—or in transgenic plants overexpressing labile and stable phytochromes (30).

The analysis of phase–response curves, not measured as a terminal response like flowering or anthocyanin formation but at the level of mRNA accumulation, will always be complicated by the fact that a second light pulse will influence the remaining level of stable Pfr and the level of newly synthesized labile phytochrome. Changes in both of these pools then rapidly affect the Pfr-induced mRNA accumulation and the phase of the running rhythm and may induce a new rhythmic accumulation of mRNA.

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