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Attenuation of Phytochrome A and B Signaling Pathways by the Arabidopsis Circadian Clock

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In higher plants, environmental cues such as light signals are integrated with circadian clock signals to control precisely the daily rhythms observed for many biological functions. We have used a fusion of the promoter of a chlorophyll *a/b* binding protein gene, *CAB2*, with firefly luciferase (*cab2::luc*) to monitor the detailed kinetics of transcription in response to photoreceptor activation in Arabidopsis. Using this marker in phototransduction and circadian-dysfunctional mutants, we studied how signals from phytochrome and the circadian clock are integrated for the regulation of *CAB2* transcription. Results from these mutant studies demonstrate that similar expression features, namely, the acute and circadian responses, are present in both etiolated and green seedlings and that the acute and circadian responses are genetically separable. We also demonstrate that persistent Pfr signaling occurs in red light–pulsed etiolated seedlings, which suggests that the circadian clock antagonizes Pfr-mediated signal transduction. Based on these genetic studies, we propose a model for the regulation of *CAB2* transcription in which individual photoreceptors and phototransduction components have been assigned to specific pathways for the regulation of discrete kinetic components of the *CAB2* expression pattern.

INTRODUCTION

Circadian rhythms are ubiquitous among eukaryotic organisms and have also been identified in some members of the cyanobacteria. These rhythms are defined as oscillations in biological function whose period length is ~24 hr. Circadian rhythms persist in the absence of environmental time cues, reflecting regulation by an endogenous pacemaker termed the circadian clock. Environmental time cues, such as light/dark (L/D) cycles, entrain the circadian clock, coordinating the activity of this endogenous oscillator with an organism's changing environment. Overt circadian responses are the result of the interactions between the endogenous clock and exogenous time cues.

In plants, as in most circadian systems, light is a primary exogenous timing cue for entraining circadian activity to the environmental day and night cycle and functions by resetting the phase of the circadian clock (Giuliano et al., 1988; Nagy et al., 1993). Light also modulates both the period and amplitude of circadian oscillations (Millar et al., 1995b). Models describing the interactions between light and the circadian clock in plant systems have been proposed based on observations of the effect of different light conditions on the oscillations in chlorophyll *a/b* binding protein (*CAB*) gene expression in both green and dark-grown (etiolated) tissue (Kay and Millar, 1992; Kay, 1993; Anderson and Kay, 1997). In these models, light signals mediated primarily by the phytochrome family of photoreceptors regulate *CAB* expression both directly, via phototransduction pathways that positively regulate the level of *CAB* expression, and indirectly, via phototransduction pathways that regulate the phase and period of the circadian oscillator. The circadian oscillator in turn regulates the timing of *CAB* gene expression. The "gating hypothesis" (Kay and Millar, 1992) proposes that the clock functions as a negative regulator of *CAB* gene expression, confining the ability of light to induce *CAB* expression to certain times during the day.

Previous studies have revealed that similar features of Arabidopsis *CAB2* expression, namely, acute responsiveness to light and high-amplitude circadian oscillations induced by light, are evident in etiolated and dark-adapted plants pulsed with light and in green seedlings under L/D cycles. The conservation of these regulatory features under different growth conditions suggests that similar regulatory pathways and mechanisms for the integration of phytochrome- and

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circadian clock–mediated signals function in etiolated, dark-adapted, and L/D-grown seedlings.

For example, in etiolated tobacco seedlings, *CAB2* expression oscillates with a very low amplitude and a period of ~30 hr (Millar et al., 1992a; Anderson et al., 1994), and in etiolated Arabidopsis, a cycle with a long period may be evident before light treatment (Millar and Kay, 1996). Brief red light treatment induces *CAB2* expression with a characteristic waveform consisting of a transient acute response peaking 2 to 4 hr after the light pulse, followed by several cycles of a circadian rhythm with a period of 30 hr or more (Millar et al., 1992a; Kay, 1993; Anderson and Kay, 1997). The red light induction of *CAB2* expression is attenuated by subsequent far-red illumination, demonstrating that phytochrome mediates this response (Chory et al., 1989; Kay, 1993).

The Arabidopsis *CAB2* gene is expressed rhythmically with a high-amplitude oscillation in green plants grown under L/D cycles. *CAB2* transcription begins to increase before the onset of light to a maximum in the middle of the light period and begins to decline before the transition to dark to a minimum in late evening. These changes in the rate of *CAB2* transcription in anticipation of the light-to-dark and dark-to-light transitions demonstrate cyclic control of *CAB2* transcription by the circadian clock. In addition, both the acute response to light and circadian regulation are evident under L/D cycles as bimodal peaks of *CAB2* expression observed at high time resolution (Millar and Kay, 1996).

In plants transferred from L/D to continuous light, the high-amplitude oscillation in *CAB2* transcription persists with a period of ~24 hr. The observation that *CAB2* oscillates in continuous light, a condition under which phytochrome is constantly activated, suggests a gating mechanism whereby the circadian clock antagonizes the light signal. Upon transfer from L/D to constant darkness, the high-amplitude oscillations in *CAB2* transcription dampen rapidly to a low level, and the period of the oscillations lengthens to >30 hr. Subsequent light treatment of such dark-adapted plants induces *CAB2* expression with a pattern similar to that in red light–pulsed etiolated seedlings (Millar and Kay, 1996). Both the magnitude and kinetics of the acute response correlate with the phase of the circadian oscillation observed in dark-grown control plants (Millar and Kay, 1996) and provide evidence that the signals mediating the acute response to phytochrome activation are attenuated by the circadian clock.

Phytochrome is encoded by a small family of genes that are differentially expressed and encode proteins with differential stability (Quail, 1994). These operationally distinct phytochrome holoproteins (phy) may have specialized or overlapping roles in the light regulation of plant processes (Furuya and Schafer, 1996), including circadian rhythmicity. Mutants with reduced levels of all phytochrome species (Chory et al., 1989) and type-specific phytochrome mutants (Sun and Tobin, 1990; Reed et al., 1994) have been used previously to examine the role of phytochromes in the induction of *CAB* expression. However, the time courses of *CAB* gene expression reported in these studies were not of sufficient duration or temporal resolution to be able to address precisely the question of how individual phytochromes contribute to the full complex regulation of *CAB* transcription.

In this study, we have advanced our understanding of the interactions between phytochromes and the circadian system by dissecting their respective inputs to the regulation of *CAB2* gene transcription. We have used the luciferase (*luc*) gene as a noninvasive reporter of gene expression that can be assayed at high temporal resolution over long experimental time courses. Bioluminescence from plants containing a *cab2::luc* fusion faithfully recapitulates the regulation of *CAB2* transcription, including induction by light and circadian rhythmicity (Millar et al., 1992b, 1995b). The *cab2::luc* marker has been crossed into a panel of Arabidopsis phototransduction and circadian-dysfunctional mutants, including type-specific phytochrome null mutants, and into lines overexpressing either phytochrome apoproteins (PHY) A or B. We have determined the contribution of the signal transduction components defined by these mutants and overexpression lines to light induction and circadian rhythmicity of *CAB2* expression in etiolated and green seedlings on an unprecedented, quantitatively comparative basis.

These genetic data establish that similar regulatory mechanisms function to regulate *CAB2* expression in both etiolated and green seedlings, although the relative importance of some of these regulatory features varies with age and developmental state. Moreover, our results allow us to assign individual phytochrome photoreceptors and downstream phototransduction components to specific pathways and DNA–protein interactions for the regulation of *CAB2* expression. In addition, we examined the kinetics of red light–induced changes in phy abundance in etiolated tissue in the context of the *CAB2* expression pattern. The results from these immunological studies, together with the gene expression patterns in the various mutants, provide evidence that the circadian clock gates the oscillations in *CAB2* expression by antagonizing persistent Pfr-mediated signals.

RESULTS

Distinct and Overlapping Roles for Type-Specific Phytochromes in the Acute and Circadian Clock Responses in Etiolated Seedlings

In etiolated wild-type Arabidopsis Landsberg *erecta* (L*er*), *cab2::luc* was expressed at a low basal level, and a red light pulse induced multiple peaks in bioluminescence, as shown in Figure 1A, in a pattern similar to that observed in other Arabidopsis ecotypes (Millar and Kay, 1996) and in tobacco (Anderson et al., 1994; Anderson and Kay, 1995). An acute response occurred with a peak in *cab2::luc* expression level 2 hr after the red light pulse, which subsequently fell to a

trough 6.5 to 8 hr after treatment. A red light–induced circadian clock response was detected thereafter as a long period oscillation (~34 hr) in *cab2::luc* expression, with a clear peak at 15.5 hr and a much weaker peak 50 hr after the red light pulse.

The ratio of the *cab2::luc* expression level at the first clock-regulated peak to that at the peak of the acute response (peak 2/peak 1 ratio) has been used previously to characterize the activation of *CAB2* expression by phytochrome (Anderson and Kay, 1995). In wild-type tobacco, the peak 2/peak 1 ratio is ~2 in lines containing full-length or 5′-deleted *CAB2* promoter sequences fused to *luc*. The ratio increases in lines containing a mutation in the repeated GATA-containing binding site within the *CAB2* promoter for the phytochrome-responsive transcription factor, CGF-1, which appears to be identical or closely related to GT-1 (Hiratsuka et al., 1994; Teakle and Kay, 1995). Moreover, GT-1 has been shown to be required for light-regulated expression of the pea gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS-3A*) (Lam and Chua, 1990). Here, we refer to the factor(s) that binds to the GATA motif in the *CAB2* promoter collectively as CGF-1/GT-1. The increase in the peak 2/peak 1 ratio observed in the CGF-1/GT-1 binding site mutant is due to a quantitative reduction in the acute response to phy activation (Anderson and Kay, 1995). In wild-type L*er*, by comparison, the peak 2/peak 1 ratio is 1.3 ± 0.1 (mean ± SE).

To characterize the roles of individual phy species in *CAB2* expression, we assayed type-specific null mutants for phytochrome A (*phyA*; Reed et al., 1994) and phytochrome B (*phyB*; Reed et al., 1993; Quail et al., 1994) for red light induction of *cab2::luc* expression in etiolated seedlings, and the results are presented in Figure 1B. In the *phyA* mutant, the acute response was attenuated ~50% relative to that in the wild type (Figure 1B). But there was no discernible effect of the *phyA* mutation on the level of *cab2::luc* expression of the circadian clock response. As a result of the differential contribution of phyA to the acute and circadian clock–regulated components of *CAB2* expression, the peak 2/peak 1 ratio was increased to 2.6 ± 0.4 in the *phyA* mutant. Therefore, light-labile phyA contributed solely to the acute response in wild-type seedlings.

In contrast, both the acute response and the circadian clock response were attenuated in the *phyB* mutant ~50% (Figure 1B). Furthermore, the acute response was attenuated in *phyB* to the same extent as in the *phyA* mutant, and the combined fold induction of *cab2::luc* expression, over the preflash level, observed in *phyA* and *phyB* (20.0 ± 2.0 and 20.9 ± 4.9, respectively) was sufficient to account for the fold induction of expression observed in the wild type (43.9 ± 7.4). Phytochromes A and B therefore contributed additively to the acute response. In *phyB*, levels of the clock-regulated peaks of *cab2::luc* expression were also reduced, indicative of a distinct role for phyB in the clock-regulated response. The peak 2/peak 1 ratio was maintained in *phyB* at a level close to that in the wild type (0.9 ± 0.1 and 1.3 ± 0.1, respectively) and is evidence that in the wild type, phyB contributes to an equal extent to both components of the *CAB2* expression pattern.

We next examined the *cab2::luc* expression pattern in the *phyA phyB* double mutant (*phyA-201 phyB-5*; Reed et al., 1994) to determine whether phytochromes A and B are sufficient for red light induction of the acute response and to determine what contribution, if any, phytochromes C, D, and E make to *CAB2* expression. *cab2::luc* expression was dramatically attenuated over the entire time course in *phyA phyB* (Figure 1C and replotted on an expanded scale in Figure 1E). The mean unadjusted preflash expression level in *phyA phyB* was approximately one-half of that in the wild type (data not shown), demonstrating that phyA and phyB modulate the *CAB2* expression level in dark-grown seedlings before light treatment. Red light induced *cab2::luc* expression in the *phyA phyB* mutant at 2 hr after the light treatment to <20% of the level in the wild type. Furthermore, the expression waveform was altered in this mutant such that there was no subsequent trough in the *cab2::luc* level. The dramatically diminished acute response in *phyA phyB* was further reflected in the increase in the peak 2/peak 1 ratio to 3.2 ± 0.4. Thus, phytochromes C, D, and E did not appear to contribute significantly to the acute component of *CAB2* expression. The first subsequent clock-regulated peak in *cab2::luc* expression, however, was evident in *phyA phyB*, suggesting that phytochromes C, D, and/or E contributed solely to the circadian component of the expression pattern.

CGF-1/GT-1 Mediates Phytochrome A and B Induction of *CAB2* Expression

The *CAB2* expression pattern in *phyA phyB*, with a low level of expression, little or no acute response, but an evident clock-regulated response, is very similar to the expression pattern observed in tobacco seedlings containing a *cab2::luc* construct with a mutation in the binding site for CGF-1/GT-1 (Anderson and Kay, 1995). To confirm the role of CGF-1/GT-1 in the regulation of *CAB2* expression in Arabidopsis in vivo, we transformed the −199/+1 wild-type and CGF-1/GT-1 mutant (G3M) versions of the *cab2::luc* construct into the Columbia (Col) ecotype, and the transformants were assayed for red light–induced *cab2::luc* expression. As in tobacco, a mutation within the CGF-1/GT-1 binding site diminished expression level and abolished the acute response but did not eliminate the clock-regulated component of *CAB2* expression in Arabidopsis (Figure 1D and replotted on an expanded scale in Figure 1E). Similar to the expression pattern in the *phyA phyB* double mutant, *cab2::luc* expression in the G3M mutant was red light–induced 2 hr after the light treatment to <10% of the level observed in the wild type, indicating almost no acute response and an attenuated circadian clock component. The observation that the CGF-1/GT-1 *CAB2* promoter mutation phenocopied the expression pattern observed in the genetically defined *phyA phyB* mutant is

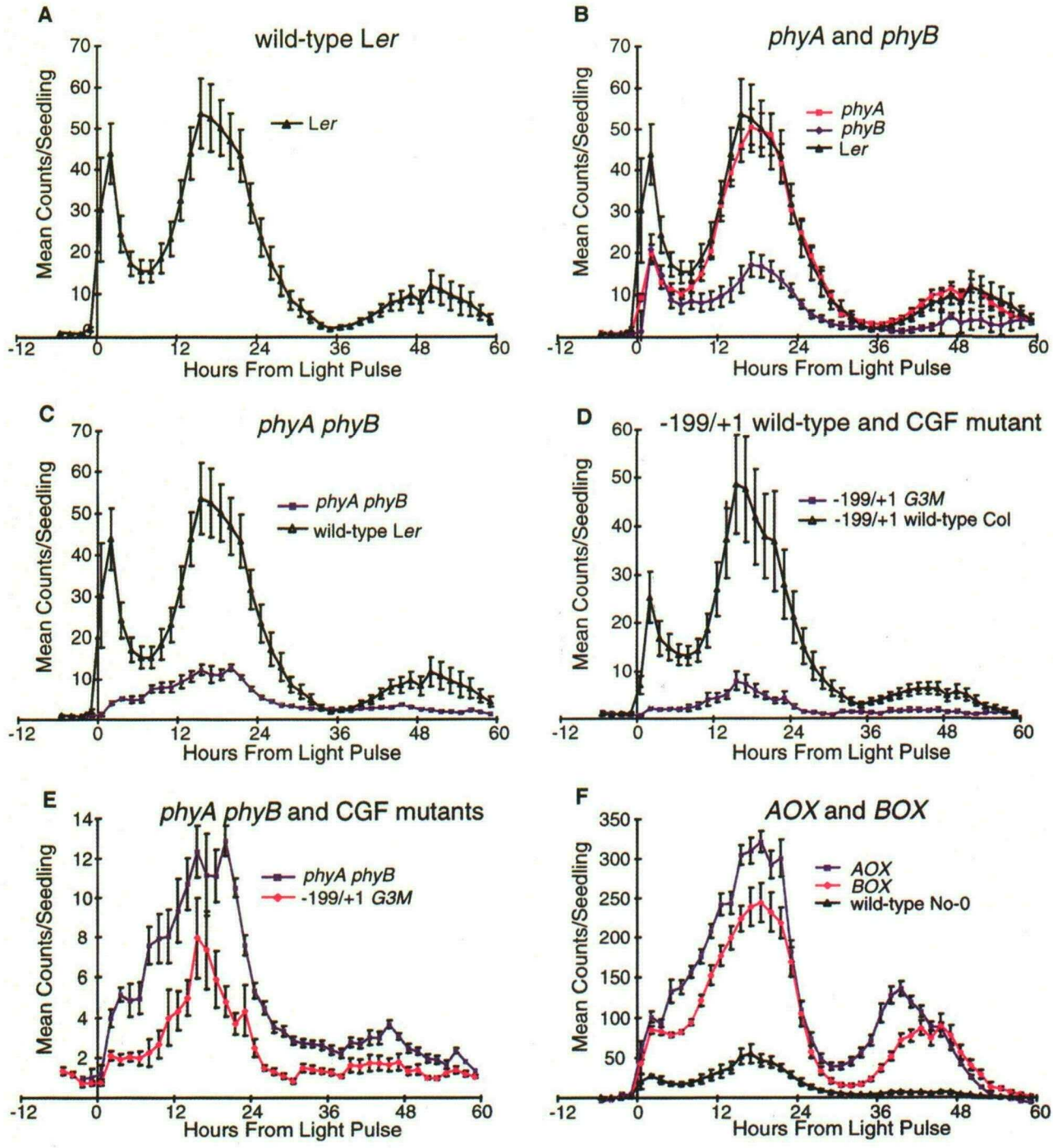

Figure 1. The Contribution of Phytochromes to the Acute and Circadian Clock–Regulated Components of *CAB2* Gene Expression in Etiolated Seedlings.

Luminescence from ~50 etiolated *cab2::luc* seedlings, which received a 2-min red light pulse at time 0 hr, is reported as mean counts per seedling for a 25-min sampling period. Arithmetic means for each time point ±SE are shown.

(A) Wild-type L*er* (black triangles), mean for pooled F_3 generation, $n = 8$. The wild-type expression time course is replotted (triangles) in **(B)** and **(C)** and in Figure 4A for comparison.

(B) *phyA-201* (pink squares), mean for four F_3 lines, $n = 28$; *phyB-5* (blue diamonds), mean for eight F_3 lines, $n = 8$.

(C) *phyA-201/phyB-5* (blue squares), mean for nine F_3 lines, $n = 9$.

strong evidence that CGF-1/GT-1 mediates phytochrome A and B induction of *CAB2* expression.

Overexpression of Phytochrome A or B Alters the Induction Pattern of *CAB2*

The cauliflower mosaic virus 35S–*PHY* overexpression lines AOX and BOX, respectively, were used to test the effect of an increased abundance of either the oat PHYA or Arabidopsis PHYB proteins on *cab2*::*luc* expression in red light–pulsed etiolated seedlings. Overexpression of either phytochrome resulted in progressively higher oscillating *cab2*::*luc* expression levels relative to that in the wild type over the entire time course, as shown in Figure 1F. The mean preflash *cab2*::*luc* levels, before normalization (data not shown), were elevated in AOX and BOX (17 ± 2-fold and 7.7 ± 1-fold, respectively) over that in the wild type. The increased content of Pr, the red light–absorbing form of phy, in the overexpression lines may increase sensitivity to the light generated by the luciferase reaction itself before the red light pulse, resulting in the generation of Pfr levels sufficient to drive higher levels of *cab2*::*luc* expression. Alternatively, a higher level of zygotic Pfr in the overexpression lines may increase *cab2*::*luc* levels before light input. It may be possible to distinguish between these alternative hypotheses by comparison of the preflash *CAB2* mRNA levels in the overexpression and wild-type lines not catalyzing the bioluminescent luciferase reaction.

Red light induced *cab2*::*luc* expression 2 hr after treatment to a greater extent in both AOX and BOX than in the wild type (Figure 1F). However, there was little or no trough in *CAB2* expression after the acute increase 2 hr after the red light pulse in either overexpression line. Overexpression of either PHYA or PHYB also caused, for the most part, similar changes in the waveform of the circadian clock response relative to that in the wild type. The fold-induction *cab2*::*luc* levels for the clock-regulated peaks in both overexpression lines were progressively higher than those in the wild type, although they still dampened in amplitude with time. In AOX, the second peak was induced sixfold and the third peak ~19-fold over that in the wild type. In BOX, the second peak was induced fourfold and the third peak ~13-fold over that in the wild type. Thus, it is clear that phytochrome levels were not saturating in the wild type with respect to the level of *CAB2* expression for either the acute or circadian clock–controlled responses. Indirect effects of phytochrome overexpression on *CAB2* expression levels such as increased growth rate and/or accelerated development cannot be discounted. Most importantly, the circadian clock was still able to override these very high levels of *CAB2* transcription and gate the phytochrome-mediated expression of the circadian response in the overexpression lines.

One change in waveform was specific to oat phytochrome A overexpression: the period of the oscillations in AOX was shortened to 22.5 hr compared with 28.5 hr in both BOX and the wild type. This period-shortening phenotype in etiolated AOX seedlings may reflect a functional difference between the phytochrome A proteins from monocots and dicots with respect to period length. However, in green seedlings of both AOX and BOX, the period of the circadian oscillations in *cab2*::*luc* expression was shortened compared with that in the wild type (D.E. Somers and S.A. Kay, unpublished results). Furthermore, no period lengthening was detected in mutant seedlings lacking the endogenous Arabidopsis phytochrome A when assayed for red light induction in etiolated tissue (Figure 1B) or in *phyA* mutant seedlings under continuous light (D.E. Somers and S.A. Kay, unpublished results). These results suggest that in the wild type, phyA levels may normally be too low to allow its participation in determining period length, but if sufficiently high levels are maintained, signaling to the clock can occur. At present, however, we cannot rule out that the effect is due to expression of heterologous PHYA.

Role of Phytochrome A and B Signaling Pathways in Green Seedlings

We extended our analysis by examining the effects of L/D cycles on the *cab2*::*luc* waveform in green seedlings. Millar and Kay (1996) have shown that the *cab2*::*luc* rhythm in L/D cycles can be dissected into an acute response and a cyclic clock–controlled response. The phytochrome-deficient and overexpressing lines provide an opportunity to examine the contribution of these photoreceptors to the establishment and maintenance of the characteristic waveform observed in the wild type. The relative contributions of the phytochromes to the generation of the acute response in light-grown plants can be compared with their roles in mediating the red light–elicited acute response described above for

Figure 1. (continued).

(D) −199/+1 G3M *cab2*::*luc* (blue squares), mean for 11 T_2 lines, $n = 11$; −199/+1 wild-type *cab2*::*luc* (triangles), mean for four F_3 lines, $n = 24$. Both reporter constructs are in the Columbia (Col) ecotype.

(E) *phyA-201 phyB-5* (blue squares), mean for nine F_3 lines, $n = 9$ (replotted from panel **[C]**); −199/+1 G3M *cab2*::*luc* (pink diamonds), mean for 11 T_2 lines, $n = 11$ (replotted from **[D]**; note the change in the y-axis scale).

(F) AOX (blue squares), mean for five F_4 lines, $n = 15$. BOX (pink diamonds), mean for two F_5 lines, $n = 6$; wild-type Nossen (No-0; black triangles), mean for pooled F_2 lines, $n = 3$.

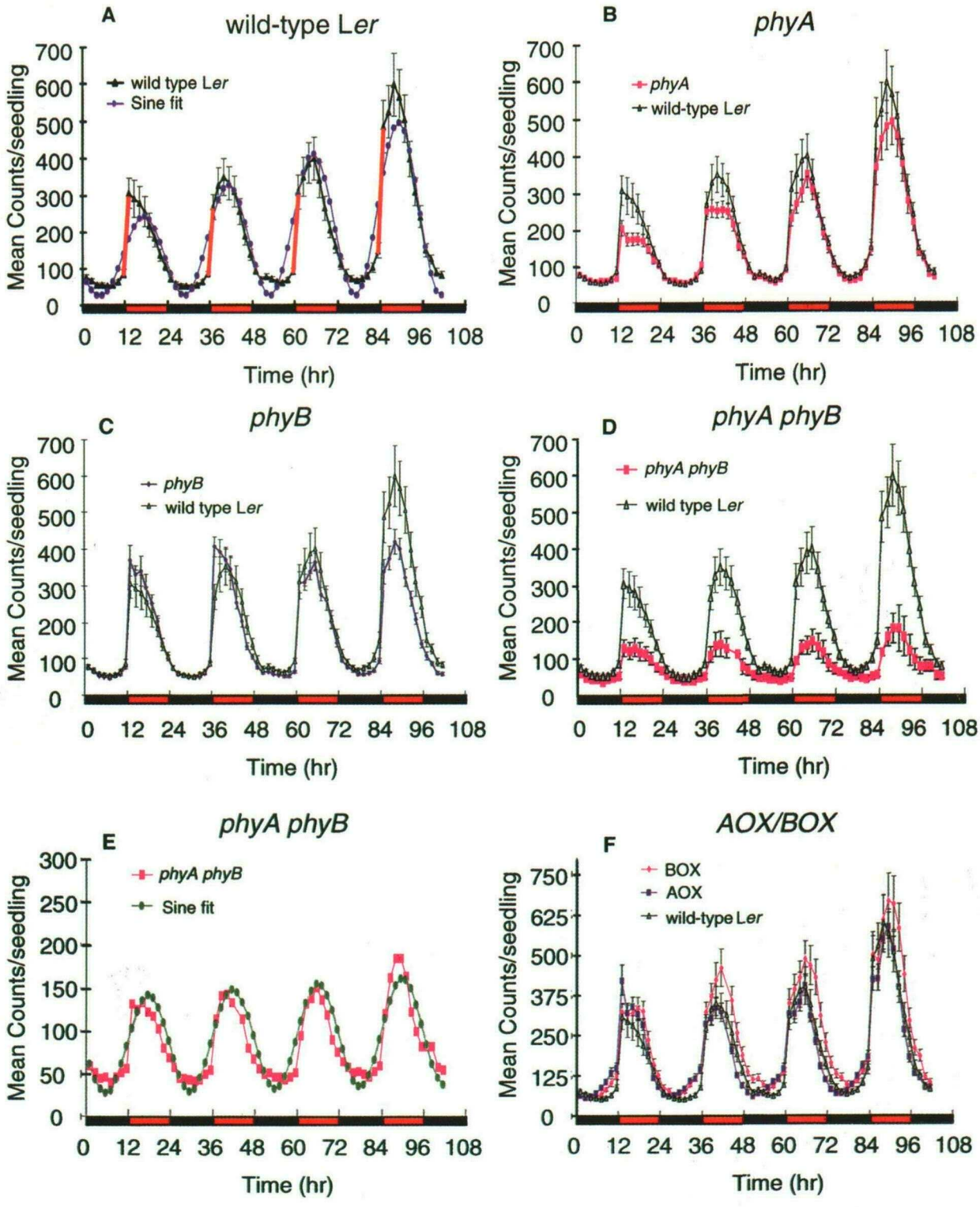

Figure 2. The Contribution of phy to the Acute and Circadian Clock–Regulated Components of *CAB2* Gene Expression in Light-Grown Seedlings.

etiolated seedlings. Thus, the dynamics of phy photoreceptor utilization in the control of *CAB2* expression throughout development can begin to be addressed.

Figure 2A shows the waveform of *cab2::luc* expression in wild-type seedlings grown under 12/12 red light/dark cycles. It is characterized by a threefold increase in *cab2::luc* expression within 1 hr after each dark-to-light transition (Figure 2A, red highlights). A modified sine wave, optimized as a mathematically derived best fit of the data, has been superimposed over the results to approximate the waveform expected from these data if only the circadian clock were gating *CAB2* expression. Over the 4-day time course, the acute rise in *cab2::luc* expression at lights-on always caused a marked divergence of the data trace from the gradual increase of the predicted curve. In contrast, after the late morning peak in *cab2::luc* expression, the data closely paralleled the falling portion of the idealized sine wave. These data strongly support the hypothesis that a clock-controlled pattern of *CAB2* expression is briefly modified by a separable, acute initial response to light received after an extended dark period.

Deficiencies in either phyA or phyB had weak effects on the overall *cab2::luc* waveform in red light/dark cycles (Figures 2B and 2C). Although *cab2::luc* expression in younger seedlings (7 days old; first 24 hr) was slightly more diminished in the *phyA* mutant compared with that in the wild type, a strong acute response was still present, relative to expression levels later in the day. During the remaining course of the experiment, both the *cab2::luc* expression levels and the acute response of the *phyA* mutant were comparable to the pattern in the wild type (Figure 2B). Similarly, the *phyB* mutant maintained a robust responsiveness to red light and overall waveform that is essentially identical to the wild type throughout the assay period (Figure 2C).

In contrast, developmental effects on the magnitude of the acute response were most apparent in the *phyA phyB* mutant background (Figure 2D and replotted on an expanded scale in Figure 2E). In *phyA phyB*, the absolute expression levels were two to three times lower than in the wild type, although the leaf area of the mutant was also reduced, making a meaningful direct comparison between this mutant line and the wild type difficult. However, *cab2::luc* expression in 7-day-old light-grown *phyA phyB* seedlings attained the peak expression of the day within the first hour after lights-on, just as in the wild type. This rapid change in *cab2::luc* expression within the first hour of lights-on makes clear that an acute response was still present in the mutant. With increasing age, the magnitude of the acute response in the wild type was maintained or was increased slightly by the end of sampling. In contrast, the extent of the acute rise in *cab2::luc* activity in the mutant, relative to daily peak expression, diminished as the plants aged, even as peak expression level remained essentially unchanged (Figure 2E). This was particularly clear during the last 3 days of sampling, when the waveform closely tracked the sine wave predicted to be the best fit of the data if only the clock were gating transgene expression (Figure 2E).

These results strongly suggest that in green seedlings, phyA, phyB, and at least one other phytochrome are involved in mediating the acute response to red light early in photomorphogenesis. As seedlings age, the relative contribution of phyA and phyB to the maintenance of the acute expression of *CAB2* grows and that of other phytochromes lessens. In contrast, the clock-regulated expression of *CAB2*, although much reduced in peak expression level and amplitude in the *phyA phyB* background, relies on the activation of one or more of the remaining three phytochromes.

Overexpression of oat PHYA or Arabidopsis PHYB had little effect on the magnitude of the acute response relative to the wild type (Figure 2F). Factors other than phy abundance appeared to limit the magnitude of the acute response to red light after a dark period, in strong contrast to the effects of PHY overexpression seen in etiolated tissue (Figure 1F). However, the subsequent clock-regulated rise in *CAB2* expression was greater and persisted longer in the BOX background than in either the wild type or AOX lines. Notable is the marked early rise in *cab2::luc* activity in the AOX and BOX lines in anticipation of lights-on relative to the wild type

Figure 2. (continued).

Luminescence in ~36 green 7- to 10-day-old *cab2::luc* seedlings grown under 12/12 white light/dark cycles after they were moved to red light/dark cycles 12 hr before beginning the assay (time 0) is reported as mean counts per seedling for a 25-min sampling period. Alternating black and red bars indicate 12-hr dark and 12-hr red light periods, respectively. Arithmetic means for each time point ±SE are shown.

(A) Wild-type L*er* seedlings (black triangles), $n = 15$. The wild-type time course is replotted in selected panels for comparison. The red line connects the *cab2::luc* expression level 30 min before lights on with the expression level 1 hr after lights on, defining the acute response to red light. The blue line is a modified sine wave, optimized as a mathematically derived best fit of the wild-type data, which approximates the waveform expected if only the circadian clock was gating *CAB2* expression.

(B) *phyA* (pink squares), $n = 18$; wild-type L*er* (triangles).

(C) *phyB* (blue diamonds), $n = 14$; wild-type L*er* (triangles).

(D) *phyA phyB* (pink squares), $n = 11$, replotted from panel **(D)**; wild-type L*er* (triangles).

(E) *phyA phyB* (pink squares), $n = 11$; the green line is a modified sine wave, as described in **(A)**, which approximates the waveform expected if only the circadian clock was gating *CAB2* expression in the *phyA phyB* background. Note the change in scale relative to **(D)**.

(F) BOX (pink diamonds), $n = 16$; AOX (blue squares), $n = 18$; wild-type L*er* (triangles).

(Figure 2F). This increase may be due to higher-than-normal levels of overexpressed Pfr present during the dark period, whose action was incompletely gated by the clock. In effect, this would result in an earlier onset of the "permissive" period of gating by the clock and *cab2::luc* levels rising earlier than normal. Alternatively, the period-shortening effects of overexpressing PHYA and PHYB may account for the earlier nocturnal rise. Either phytochrome, when overexpressed, shortens the free-running period in red light to significantly <24 hr (D.E. Somers and S.A. Kay, unpublished results). When grown under a 24-hr L/D cycle, these plants begin to anticipate lights-on earlier than would the wild type, which runs with an endogenous period of 24.5 hr (data not shown). Currently, we cannot distinguish between these two alternative hypotheses.

HY5 Contributes to the Circadian Clock–Regulated Response of *CAB2* Expression in Etiolated Seedlings

We assayed *cab2::luc* expression in selected phototransduction mutants to determine the contribution of signaling components that function downstream of the phytochrome photoreceptor to *CAB2* gene expression. The *hy5* mutant, identified by its long hypocotyl phenotype under growth in the light (Koornneef et al., 1980), is insensitive to red and far-red light and to a lesser extent to blue light. Although *hy5* contains normal levels of phytochrome and is not likely to be a photoreceptor mutant, this mutant appears to have reduced signal transduction capability (Koornneef et al., 1980; Chory and Susek, 1994). In a previous study, no defect in phy regulation of *CAB* gene expression was detected in *hy5* when *CAB* mRNA levels were examined at low time resolution during the early portion of the expression pattern corresponding to the acute response (Sun and Tobin, 1990). The results presented in Figure 3A confirm that observation. HY5 is not a component of the signaling pathway regulating the acute response. However, the phytochrome-induced, clock-regulated component of *cab2::luc* expression is attenuated in *hy5* by 50% relative to the wild type. This suggests that the HY5 protein participates in signal transduction pathways that contribute only to the clock-regulated component of *CAB2* gene expression.

ELF3 Is Necessary for the Circadian Clock–Regulated Response but Not the Acute Response of *CAB2* Expression in Etiolated Seedlings

elf3 is a photoperiod insensitive, early flowering mutant that is defective in signal transduction pathways responsive to blue light and to other wavelengths (Zagotta et al., 1996). *elf3* also displays conditional circadian dysfunction. No obvious defects in circadian rhythmicity are apparent in green *elf3* seedlings under continuous darkness or in etiolated *elf3* seedlings before a red light pulse when assayed in the *supernova* background, which increases the level of *CAB2* expression and improves our ability to detect *CAB2* cycling in etiolated tissue (Hicks et al., 1996). However, the circadian oscillations in both *cab2::luc* levels and leaf movements are absent under continuous light in *elf3* (Hicks et al., 1996). Based on this conditional arrhythmic phenotype in multiple clock outputs, it has been proposed that the circadian clock is mostly intact in *elf3*, but aberrant transduction of light signals to the clock occurs in the absence of *ELF3* function (Hicks et al., 1996).

Because *elf3* displays an arrhythmic phenotype in continuous light, it is possible that a conditional circadian defect is also manifest in etiolated *elf3* seedlings given a brief red

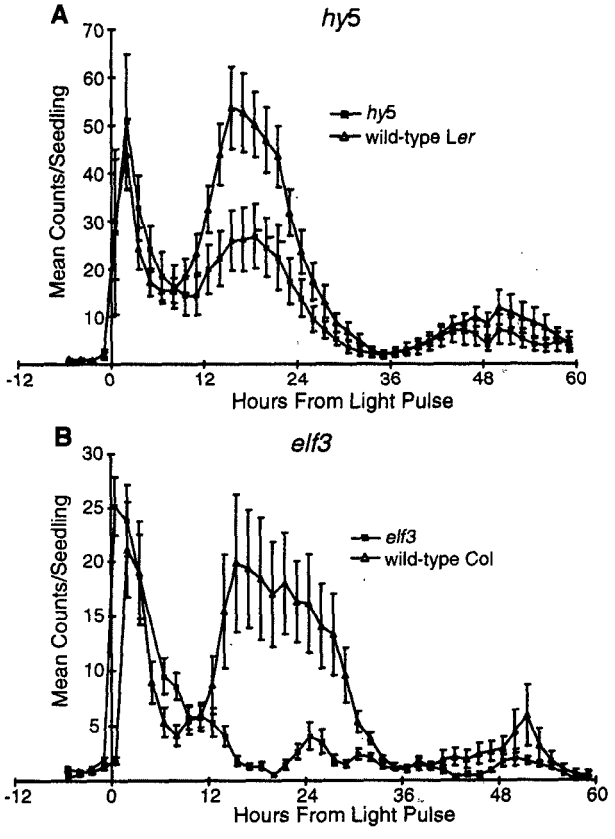

Figure 3. The Contribution of Phototransduction Pathways Defined by the *hy5* and *elf3* Mutants to the Acute and Circadian Clock–Regulated Components of *CAB2* Gene Expression in Etiolated Seedlings.

Luminescence in etiolated *cab2::luc* seedlings was determined as described in Figure 1 and in Methods.

(A) *hy5* (blue squares), mean for an F_4 line, $n = 6$; wild-type *Ler* (triangles), mean for pooled F_3 lines, $n = 8$.

(B) *elf3* (blue squares), mean for two F_3 lines, $n = 6$; wild-type Col (triangles), mean for an F_3 line, $n = 3$.

light pulse. We therefore examined the *cab2*::*luc* expression pattern in this mutant, and the results are shown in Figure 3B. The effects of the *elf3* defect were evident immediately after the light pulse. The acute response persisted in *elf3*, and a red light treatment induced *cab2*::*luc* expression to a level similar to that in the wild type but with altered induction and decay kinetics. The induction kinetic in *elf3* was substantially more rapid than in the wild type, with the peak of the acute response occurring in *elf3* just 0.5 hr after the light pulse. One possible explanation for an accelerated induction kinetic in *elf3* is that this mutation may in some way enhance phototransduction activity. However, in the phytochrome overexpression lines, in which light signal input was increased, the induction kinetic was not accelerated and the *cab2*::*luc* expression levels at 0.5 hr were only one-half of that at 2 hr (Figure 1F). This suggests that the accelerated induction kinetics in *elf3* is not due to increased phototransduction activity but rather results from derepression of *CAB2* expression after a light pulse. Consistent with this hypothesis, the decay of the acute response was also slowed somewhat in *elf3* and required 9 hr relative to 6 hr in the wild type to decline to a transitory minimum. The *elf3* mutation had a similar effect in green seedlings under L/D cycles, in which *cab2*::*luc* expression showed an accentuated acute response, and the kinetics and even the magnitude of induction appeared to be enhanced relative to that in the wild type (Hicks et al., 1996).

In comparison, the circadian clock response was severely disrupted in *elf3.* The *elf3* mutation all but eliminated any long-period oscillations in *cab2*::*luc* expression, although there is some evidence of very low amplitude oscillations with a period of ~12 hr (Figure 3B). A brief red light treatment is therefore sufficient to reveal a conditional circadian clock defect in etiolated *elf3* seedlings, similar to that observed in green seedlings transferred to continuous light. The observation that, subsequent to the acute response, light did not induce long-period oscillations in *elf3* leads us to suggest that after a red light treatment, the clock continuously antagonizes light input to *CAB2* expression and halts the cyclic expression of the *CAB2* gene. Moreover, these results demonstrate that normal clock function is not required for red light induction of the acute response, although the clock and/or ELF3 appear to contribute to the kinetics of the acute peak in *CAB2* expression in response to light input.

Light Regulates the Abundance of Total Phytochrome A and Phytochrome B in Etiolated Wild-Type Arabidopsis Plants

The studies presented above, either by the use of mutants deficient in one or more phy species or by the overexpression of phytochrome proteins, have revealed dramatic effects of altering phytochrome abundance on *CAB2* expression. In particular, the period-shortening effect of PHYA overexpression observed as late as 40 hr after the red light pulse and the increasing fold induction of *cab2*::*luc* expression above that in the wild type are evidence that significant levels of active oat phyA are likely still to be present throughout the expression time course. Therefore, it was of interest to investigate the abundance of phy in relation to the *cab2*::*luc* expression time course in etiolated wild-type, AOX, and BOX seedlings pulsed with red light. Total phyA and phyB levels were analyzed in samples collected at characteristic points over the *cab2*::*luc* expression time course: just before the red light pulse, at the peak of the acute response and first and second clock-controlled oscillations, and at the minimum of the first and second troughs (Figure 4A).

Quantitation of total phy abundance in wild-type seedlings over the *CAB2* time course (Figure 4B) revealed that by 2 hr postflash, the phyA level was reduced >75%. phyA levels then slowly reaccumulated over the next 48 hr in the dark. Somewhat surprisingly, the level of phyB decreased >50% after a red light pulse and slowly recovered over the remainder of the time course. The red light–mediated decrease in total phyB abundance in etiolated seedlings has not been reported previously.

The AOX lines overexpress the heterologous oat PHYA protein in the background of the endogenous Arabidopsis phyA protein. However, a monoclonal antibody that recognizes just the monocot-specific form of phytochrome was used to detect only the oat phyA protein. The phyA levels determined for AOX therefore contain no contribution from the changing levels of the endogenous Arabidopsis phytochrome A. The phytochrome A and B proteins were present at constitutive steady state levels in their respective overexpression line (Figure 4C). It is not unexpected that no change in total phyA or B abundance was detected in etiolated seedlings of the overexpression lines pulsed with red light, because no differences in phytochrome level were previously detected in these lines between dark- and light-grown seedlings (Boylan and Quail, 1991; Wagner et al., 1991).

Pfr-Mediated Signal Input to the *CAB2* Promoter Persists in Etiolated Seedlings Overexpressing Oat Phytochrome A

Although no differences in total phy abundance were detected in the overexpression lines, it is the Pfr form of phytochrome that mediates the induction of *CAB2* expression. The active Pfr form of phytochrome is much more rapidly degraded in vivo than is the inactive Pr form (Vierstra, 1993). Therefore, we determined the levels of Pfr in etiolated AOX seedlings over the *CAB2* expression time course. Figure 5 shows that partial in vitro proteolysis of protein extracts from AOX with subtilisin produced different proteolytic fragments when phyA was in the Pr or Pfr form. Because the monocot-specific phyA antibody 1.9B5A did not yield reproducible differential proteolytic fragments (results not shown), the phyA proteins were detected in this experiment with the monoclonal antibody 073D, which recognizes both monocot and dicot forms of phyA. Therefore, the proteolysis patterns

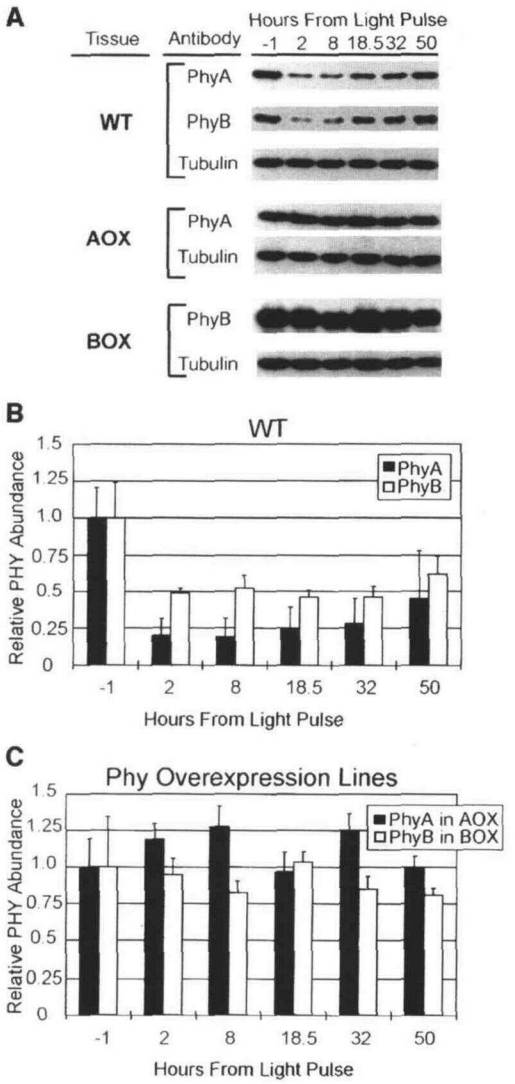

Figure 4. The Contribution of Total phy Abundance to the Regulation of *CAB2* Expression in Etiolated Seedlings.

(A) Immunoblot analyses of phy abundance over the *cab2::luc* expression time course in etiolated wild-type (WT) and 35S–*PHY* overexpression lines (AOX and BOX) receiving a red light pulse. Crude protein extracts prepared for the determination of phytochrome levels were probed for phyA with the monocot phytochrome A type-specific antibody, 1.9B5A; for phyB, a mixture of three monoclonal antibodies, B1, B7, and B8, was used; and for tubulin, with the β-tubulin–specific antibody, Tu27. Representative blots are shown.

(B) Quantitation of the relative abundance of phyA (black bars) and phyB (open bars) proteins in etiolated wild-type No-0 *cab2::luc* seedlings pulsed with red light. Phytochrome levels were calculated relative to the level in the preflash sample at −1 hr and were normalized for protein loading to tubulin levels. Plotted are the mean phy levels for two sets of crude protein extracts from independent experiments run on four replicate immunoblots each. The error bars represent one standard error of the mean.

shown are for the pool of both endogenous and heterologous phyA proteins.

A proteolytic fragment predominant in the Pfr form, indicated in Figure 5 with an asterisk, was used for the quantitation of Pfr levels over the *cab2::luc* time course. The level of this Pfr-specific band was normalized to the level of a band present in equal amounts in both the Pfr and Pr forms (filled circle). At 2 hr after the red light pulse, Pfr levels were induced fourfold above the preflash level; they decayed to approximately twofold above preflash level by 18.5 hr after the red light pulse, and remained at least twofold above the preflash level for the remainder of the time course. The Pfr degradation rates for monocot phytochromes have been shown to be, in general, slower in transgenic dicot plants than in the parent monocot plants, and Pfr half-lives on the order of 4 hr have been reported for oat phyA in transgenic dicots under continuous red light illumination (Vierstra, 1993). The Pfr levels determined in our study are indicative of a Pfr half-life longer than those reported previously. Moreover, the persistence of Pfr levels twofold above background for up to 48 hr demonstrates that the Pfr-mediated signal input to *CAB2* expression lasts over several circadian cycles. This suggests that, as for *CAB2* expression in continuous light, the circadian clock must antagonize the persistent Pfr-mediated signal input in etiolated seedlings pulsed with red light to generate the oscillations in *CAB2* expression.

DISCUSSION

Similar Phytochrome and Circadian Clock Signaling Pathways Operate in Etiolated and Green Seedlings

Several recent advances in our understanding of the circadian system in plants have come from studies using the noninvasive bioluminescent *cab2::luc* marker. These include the genetic identification of molecular components of the circadian oscillator (Millar et al., 1995a) and the identification of the genomic targets of the circadian clock (Carré and Kay, 1995) and phytochrome phototransduction (Anderson and Kay, 1995) pathways at the level of the *CAB2* promoter. We have shown here that the *CAB2* expression pattern in etiolated seedlings pulsed with red light consists of two

(C) Quantitation of the relative abundance of oat phyA (in AOX, black bars) and Arabidopsis phyB (in BOX, open bars) in etiolated *cab2::luc* seedlings of PHY overexpression lines pulsed with red light. Phytochrome levels were calculated as for **(B)**. Plotted are the mean phy levels for a single set of crude protein extracts for each line, run on three replicate immunoblots each. Similar results were obtained using protein extracts made from tissue collected in an independent red light pulse experiment. The error bars represent one standard error of the mean.

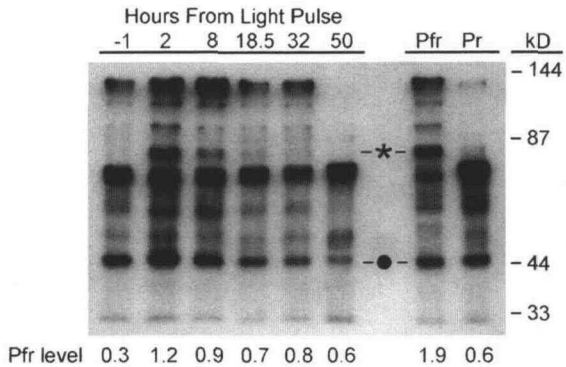

Figure 5. Changes in Pfr Abundance in a 35S–PHYA Overexpression Line over the *cab2::luc* Expression Time Course as Determined by in Vitro Proteolysis of phyA.

An immunoblot of soluble protein extracts from AOX was subjected to partial proteolysis with subtilisin and probed with 073D, an antibody that recognizes both monocot and dicot forms of the phyA protein. Before proteolysis, aliquots of the −1 hr protein extracts were given either a saturating red (Pfr) or far-red (Pr) light treatment in vitro. The Pfr level was calculated for each sample as the ratio of chemiluminescence of the Pfr-specific band (asterisk) to that for a band present at a constitutive level in the Pfr and Pr samples (filled circle).

distinct kinetic components: the acute response and the clock-regulated response. The same components can be distinguished in the bimodal peaks of *CAB2* expression in green seedlings under L/D cycles, as previously demonstrated (Millar and Kay, 1996).

We have demonstrated that the *cab2::luc* expression patterns observed in etiolated and green seedlings are affected in a similar fashion by the phototransduction mutations examined in this study (Figures 1 and 2). Thus, although the relative quantitative contribution of individual phytochrome species may vary somewhat depending on growth conditions and development, particularly in green seedlings, the complex oscillating patterns of gene expression observed under different growth conditions reflect the operation of similar regulatory mechanisms. Therefore, etiolated seedlings provide a valid system for the analysis of the phytochrome and circadian clock pathways regulating *CAB2* expression that also operate in green seedlings. In addition, the acute and circadian responses are resolved more clearly in etiolated seedlings, facilitating their analysis in different genetic backgrounds.

Multiple Genetically Defined Phytochrome Phototransduction Pathways Regulate *CAB2* Transcription

The *cab2::luc* expression patterns in the *phy* mutants (Figures 1 and 2) clearly establish that different phytochromes have both distinct and overlapping roles in *CAB2* regulation. In etiolated plants, phytochromes A and B together are sufficient and contribute equally in an additive fashion to mediate the acute response. phyB has a distinct role in etiolated seedlings to regulate positively the level of expression of the circadian oscillations, whereas phyA has no discernible role in the circadian response. The three phytochromes remaining in the *phyA phyB* double mutant make a minor quantitative contribution to the circadian response of *CAB2* expression in etiolated seedlings.

In contrast to etiolated seedlings, phytochromes A and B individually do not appear to make as significant a contribution to either the acute or the circadian response in green seedlings. Both phyA and B together contribute quantitatively to the acute response and to the amplitude of the circadian oscillations in green seedlings, as revealed in the *phyA phyB* mutant. In addition, one or more of the remaining phytochromes must also contribute significantly to the overall amplitude of the circadian oscillations and early in green seedling development to the acute rise in the *CAB2* waveform. Based on mean *cab2::luc* expression levels, the contribution from the remaining phy species (C, D, and E) is more significant in green than in etiolated seedlings.

Interestingly, normal clock function is not required to mediate the acute response, because the acute response persists, albeit with altered kinetics, in the conditionally circadian-dysfunctional mutant *elf3* (Figure 3B). Thus, as demonstrated for *CAB* expression in barley (Beator et al., 1992), we demonstrate here that the acute response of *CAB2* expression in Arabidopsis is genetically separable from the circadian clock. Similarly, light induction of the *Neurospora crassa* clock-controlled genes *ccg-1* (Arpaia et al., 1995) and *ccg-2* (Arpaia et al., 1993) does not require an intact circadian oscillator. Separable regulation by light and the circadian clock may therefore be a common feature for genes regulated by both light and the circadian clock.

Distinct functional roles for specific phytochromes in the regulation of *CAB2* expression imply that different phytochromes interact with different phototransduction pathways. The additive contribution and overlapping function of phyA and phyB to the acute response in etiolated seedlings indicate that these photoreceptors mediate light signals via initially independent pathways that ultimately converge at or before the *CAB2* promoter. In addition, the distinct regulatory role for phyB in the circadian response is evidence that this photoreceptor is also coupled to a phyA-independent phototransduction pathway branch specific to the circadian response, to which phytochromes C, D, and/or E also contribute.

The genetic evidence presented here for independent phototransduction pathways for phyA- and phyB-mediated responses supports recent microinjection studies (Kunkel et al., 1996) demonstrating different physiological roles for phyA and B in etiolated plants. In the reciprocal control model for phyA regulation of anthocyanin accumulation and chloroplast development (Neuhaus et al., 1993; Bowler et al., 1994), the Pfr form of phyA activates one or more G

proteins, which in turn activates downstream signaling pathways regulating subsets of phytochrome-mediated responses: a cGMP-dependent pathway regulating anthocyanin biosynthesis; a Ca^{2+}/calmodulin–dependent pathway regulating transcription of *rbcS*, *CAB*, and photosystem II–related genes; and a partially convergent pathway required for photosystem I–related gene expression and for complete chloroplast development. In comparison to the observed effects of microinjection of phyA, microinjection of phyB induces photosystem II–related gene expression, implying activation of the Ca^{2+}/calmodulin–dependent pathway, but does not induce the production of anthocyanins (Kunkel et al., 1996). These results suggest that phyB activates a subset of G proteins distinct from those with which phyA interacts and that the phyB phototransduction pathway must converge at some point farther downstream with the Ca^{2+}/calmodulin–dependent pathway.

Our results demonstrating that the *phyA phyB* double mutant phenocopies the expression pattern of the CGF-1/GT-1 binding-site mutant (Figures 1D and 1E) suggest that the CGF-1/GT-1 binding site in the *CAB2* promoter is the genomic target for phytochrome induction of *CAB2* gene expression and that the signal transduction pathways from phyA and phyB must converge at or before their interaction with CGF-1/GT-1 at the level of the *CAB2* promoter (see Figure 6). This conclusion is further supported by microinjection studies demonstrating that the genomic target of the Ca^{2+}/calmodulin–dependent phyA signaling pathway in the *rbcS-3A* promoter is the binding site for GT-1, box II (Wu et al., 1996). A *phyA phyB* CGF-1/GT-1 binding-site mutant could be used to confirm that the CGF-1/GT-1 binding site is the target of phyA and phyB transduction pathways, because no further reduction in expression should be observed in such a background. Another factor, CCA1, has been identified as contributing to phytochrome-regulated transcription of the *Lhcb1*3* gene, which is closely related to *CAB2* (Wang et al., 1997). It is not yet clear if this factor contributes to phytochrome or circadian clock regulation of *CAB2*, but it clearly shows that more than one DNA–protein interaction is likely to contribute to phytochrome-responsive transcription of *CAB* genes.

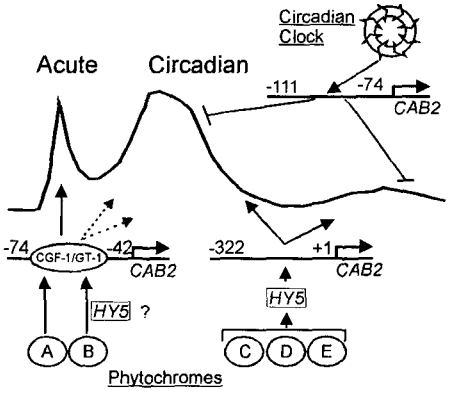

Figure 6. A Model of the Phototransduction and Circadian Clock Pathways That Regulate *CAB2* Transcription.

Phytochromes A and B signaling pathways mediate the acute response via CGF-1/GT-1. HY5 contributes only to the later circadian clock component downstream of phytochromes B, C, D, or E. The circadian clock antagonizes phytochrome-mediated induction of *CAB2* transcription to produce a cyclic transcription pattern. Numbers indicate base position relative to start of transcription.

Based on the *cab2::luc* expression pattern in etiolated seedlings of the *hy5* mutant (Figure 3A), HY5 is clearly not a component of the phyA phototransduction pathway. The HY5 protein appears to be associated strictly with the circadian response. The simplest model for placing HY5 in a phototransduction pathway(s) for the regulation of *CAB2* expression is that HY5 mediates signals exclusively in a phototransduction pathway from phytochrome C, D, and/or E (Figure 6), which also contribute only to the circadian response in etiolated (Figures 1C and 1E) and green (Figure 2E) seedlings. Because phyB also contributes to the circadian response, HY5 may also mediate signals from phyB but only as a component of a phyB phototransduction pathway distinct from the convergent phyA and phyB pathway that mediates the acute response.

The *HY5* locus has been cloned recently and encodes a member of the basic leucine zipper family of DNA binding proteins and is postulated to function as a transcription factor involved in light signal transduction (K. Okada, personal communication). Analysis of the *CAB2* promoter has identified a unique, nonpalindromic G-box site, which is bound in vivo by a heterodimeric basic leucine zipper DNA binding protein, termed CUF-1 (Anderson et al., 1994). Mutation of the CUF-1 binding site in transgenic tobacco suggests that CUF-1 functions to confer high levels of *CAB2* expression but is not required for correct kinetics of phytochrome induction or, unlike HY5, for circadian clock regulation of *CAB2* expression (Anderson and Kay, 1995). It will therefore be of great interest to determine whether HY5 interacts with the *CAB2* promoter directly, whether HY5 is a component of or interacts with CUF-1, and/or whether it is involved in the transcriptional regulation of a signal transduction component(s) that functions upstream of the *CAB2* promoter.

Changes in Total Phytochrome A and Phytochrome B Abundance Cannot Fully Account for the Decay of the Acute Response in the Wild Type

Phytochrome type specificity is clearly an important aspect of light input to *CAB2* gene expression. And as demonstrated in this study, phytochrome abundance is also an important contributor to the expression pattern in the wild type. It has been a long-standing hypothesis (Kay, 1993) that the light-labile phyA species mediates the acute response in etiolated wild-type seedlings and that the rapid decay of the acute response correlates with the rapid decay

of phyA upon conversion to the Pfr form. We have clearly demonstrated in this study that phyA as well as phyB contribute to the acute response in etiolated (Figure 1B) and green (Figure 2D) seedlings. Therefore, the acute response cannot be fully explained simply by the activation and subsequent decay of total phyA. Interestingly, light also appears to regulate total phyB abundance, although phyB levels are not reduced in etiolated seedlings to the same extent as is phyA by light treatment (Figure 4B). Although the changes in total phy abundance are largely coincident with the changes in the waveform of the acute response (Figure 1A), the activation and subsequent decline in the total steady state levels of both phyA and B do not appear to be the sole factors governing the waveform of the acute response. This is evident from the time resolution used, where the total abundance of both phyA and phyB were already at a minimum at the acute peak of transcription. This observation prompted us to measure Pfr levels rather than total phytochrome.

The Persistent Pfr-Mediated Signal Input Is Antagonized by the Circadian Clock

Extended high-amplitude *CAB* oscillations were observed previously in tobacco plants overexpressing rice PHYA upon transfer from continuous light to continuous darkness (Kay et al., 1989). It was proposed that the extended *CAB* oscillations reflected the increased Pfr half-life and abundance of rice phyA in transgenic tobacco. The possibility that the exogenous rice phyA might also interfere with the endogenous phy function could not be discounted in this earlier study.

We demonstrate here that the total phyA or phyB abundance driven by the 35S promoter in the respective overexpression lines remains relatively unchanged over the *CAB2* expression time course (Figure 4C). In comparison, the abundance of the biologically active Pfr form of phyA in the AOX line changes dramatically in response to a red light pulse. The results from this experiment demonstrate that although the total phy abundance is at a minimum 2 hr after a red light pulse, at the same time resolution, the Pfr form of phyA is at maximum abundance 2 hr after a red light pulse and decays to twofold above preflash level by 18.5 hr after the light treatment (Figure 5). In addition, the Pfr levels remain at least twofold higher than the preflash level for the remainder of the time course, demonstrating persistent Pfr signal input in the overexpression line. This result is not unexpected because the predominant phy present in the AOX line is the more stable monocot phyA.

Based on our demonstration here of persistent Pfr signaling in AOX, it is reasonable to predict that the Pfr form of the light-stable phyB species also persists over the *CAB2* expression time course in the endogenous PHYB overexpression line BOX. Importantly, the *cab2::luc* waveform is affected by PHYB overexpression in a manner similar to that in AOX. Because the BOX lines resulted in the same alterations in expression, it is unlikely that the extended high-amplitude *cab2::luc* oscillations in the AOX line result from the interference of oat phyA with the function of the endogenous phy but rather reflect persistent Pfr signaling, which is antagonized by the action of the circadian clock.

Model of the Integrated Phytochrome- and Circadian Clock–Regulated Pathways Controlling *CAB2* Transcription

Many of the interactions required for the regulation of *CAB2* expression in etiolated tissue, as defined in this study, are summarized in the conceptual model shown in Figure 6. Independent pathways from phytochromes A and B converge at or before interaction with CGF-1/GT-1 at the level of the *CAB2* promoter to induce the acute response. phyB also mediates the circadian response through a separate pathway. This phyA-independent pathway also likely involves CGF-1/GT-1, because mutation of the CGF-1/GT-1 binding site also attenuates the amplitude of the circadian response in both green and etiolated tissue (Anderson and Kay, 1995). Phytochromes C, D, and/or E contribute only to the circadian response via interaction with sequences within the first 322 bp of the *CAB2* promoter. It is possible that CGF-1/GT-1 is involved in signaling from these phytochromes because no other phy-responsive factors interacting with the minimal phy-responsive *CAB2* promoter have been functionally identified. Distinct phy species interact with distinct downstream signal molecules. Specifically, HY5 participates in signaling from phytochromes C, D, and/or E and possibly from phyB to mediate the circadian oscillations in *CAB2* expression. ELF3 appears to participate in the phy-regulated pathway controlling the circadian response. Because the acute response is altered slightly in the *elf3* mutant, a role for ELF3 in the pathway controlling the acute response cannot be fully discounted. However, the *elf3* results demonstrate that functional circadian rhythmicity is not required for the acute response and that the acute and circadian responses are genetically separable. The enhanced *cab2::luc* oscillations in the PHY overexpression lines pulsed with red light, as in green seedlings under continuous light, coupled with the demonstration here of persistent Pfr-mediated signal in AOX, are consistent with a gating mechanism in which the circadian clock antagonizes the Pfr-mediated signal to generate the oscillations in *CAB2* expression.

Implications for Further Studies

It will be of great interest to determine how the regulatory interactions determined here for the day phase–specific *CAB2* gene relate to those involved in night phase–specific gene expression, such as *CAT3* (Zhong and McClung, 1996). Questions of particular interest are: Do night-phase genes maintain either a positive or negative acute response to

light, and what role do individual phytochrome species play in the regulation of night phase–specific genes?

Finally, the detailed identification of phytochrome and circadian clock inputs into *CAB2* expression described here will serve as criteria for the characterization of novel clock mutants. Examination of the *cab2::luc* expression patterns in red light–pulsed etiolated seedlings will provide a means for distinguishing whether an altered circadian phenotype is due to a defect in light input into the system or more likely to a lesion in a component of the circadian oscillator itself.

METHODS

Plant Material and Growth

A transgenic *Arabidopsis thaliana* line containing a fusion of the chlorophyll *a/b* binding protein 2 gene promoter and firefly luciferase (*cab2::luc*) in the C24 background has been described previously (Millar et al., 1992b) and was used to cross the *cab2::luc* marker into both phototransduction mutants and phytochrome overexpression lines and into the appropriate wild-type Arabidopsis ecotypes. Lines homozygous for both the specified phototransduction trait and the *cab2::luc* marker were selected on the basis of morphological phenotype and by screening for bioluminescence. The lines are as follows: wild-type Landsberg *erecta* (Ler) and, in the Ler background, *phyA-201* (Reed et al., 1994), *phyB-5* (Reed et al., 1993; Quail et al., 1994), *phyA-201/phyB-5* (Reed et al., 1994), and *hy5* (Ci88; Koornneef et al., 1980); wild-type Nossen (No-0) and, in the No-0 background, the rice phytochrome A overexpresser (AOX, line 13k7; Boylan and Quail, 1991) and the Arabidopsis phytochrome B overexpresser (BOX, line ABO; Wagner et al., 1991). The wild-type Columbia (Col) ecotype (Millar et al., 1992b) and *elf3-1* (Hicks et al., 1996) lines containing the *cab2::luc* marker were described previously. The promoter deletion containing the first 199 bp of *CAB2* upstream sequence (−199/+1; Anderson et al., 1994) and −199/+1 containing a triple GATA motif mutation (G3M; Anderson and Kay, 1995) *cab2::luc* constructs, described previously, were transformed into the Col ecotype of Arabidopsis by vacuum infiltration (Bechtold et al., 1993; Bent et al., 1994). Lines containing the *cab2::luc* marker were selected as kanamycin-resistant plants and subsequently screened by monitoring bioluminescence. Homozygous −199/+1 wild-type *cab2::luc* lines were identified; however, the −199/+1 G3M *cab2::luc* T_2 lines are heterozygous and still segregating the bioluminescent marker. A correction factor of 1.25 was therefore applied to the *cab2::luc* expression levels determined for the −199/+1 G3M *cab2::luc* T_2 lines, allowing direct comparison to expression levels in homozygous *cab2::luc* lines.

Seeds of the *cab2::luc* lines were sterilized and plated on Petri plates containing solid Murashige and Skoog medium (Sigma) with 3% sucrose and kanamycin sulfate (50 μg/mL). For dark-grown etiolated seedlings, ~50 seeds per line were plated in a cluster contained within a plastic collar (1.5-cm lengths of clear plastic drinking straws, sterilized by soaking in 70% EtOH, dried, and inserted into the agar medium). The plates were wrapped with several layers of aluminum foil and kept at 4°C for 4 days. Germination was induced by a 15-min exposure to dim white light, and the plates were subsequently wrapped with aluminum foil and maintained in a dark box inside of a constant temperature darkroom at 22°C. The unexpanded cotyledons of the etiolated seedlings were at or near the tops of the collars at the time imaging of *cab2::luc* bioluminescence was initiated. Confinement of the seedlings within the collars increased the signal-to-noise ratio (data not shown), thus allowing more accurate quantitation of *cab2::luc* expression levels.

For light-grown plants, seeds were sown in a grid pattern and stratified for 2 days at 4°C and under dim light. Seedlings were grown under a 12-hr light/12-hr dark photoperiod in a controlled environment chamber at 22°C and under light conditions described previously (Millar et al., 1995b).

Bioluminescence Assays

cab2::luc bioluminescence was detected and quantified using an intensified CCD camera and photon-counting image processor (Hamamatsu Photonics Systems, Bridgewater, NJ). Bioluminescence imaging conditions were as described previously (Anderson et al., 1994), except that seedlings were imaged at 1.5-hr time resolution for 25 min, and *cab2::luc* expression levels are reported as mean counts per seedling. For etiolated seedlings, imaging of *cab2::luc* bioluminescence was initiated at dawn on day 5 of growth. Seedlings tested for phytochrome-responsive *cab2::luc* expression were irradiated with red light, as described previously (Anderson et al., 1994), at the indicated time and returned to constant darkness for the remainder of the experiment. Because the circadian clock gates phytochrome responsiveness (Millar and Kay, 1996), manipulations of plant material, including sowing, induction of germination, transfer to growth conditions, the initiation of imaging, and red light irradiation, were done at the same time during the day to maintain a consistent phase relationship to the clock for each replicate experiment. For light-grown seedlings, imaging of *cab2::luc* bioluminescence was initiated at dawn on day 6 of growth and continued for 5 days.

Video images were analyzed as previously described (Millar et al., 1992b), and the periods of the *cab2::luc* oscillations of the clock-regulated response in red light–pulsed etiolated seedlings were calculated manually. All *cab2::luc* expression data for the various etiolated wild-type, mutant, and overexpression lines were normalized by subtracting from each expression time course the respective mean preflash expression level minus one ($mean_{preflash}-1$), thereby setting the preflash levels for all lines to values centered at ~1. Thus, the *cab2::luc* expression levels reported for each line are indicative of the fold induction of *CAB2* expression over background. With the exception of *phyA phyB* and the AOX and BOX lines, the unnormalized preflash levels in the mutant lines were not significantly different from those in the respective wild-type lines (data not shown), and the *cab2::luc* expression levels plotted for these lines are therefore also indicative of the actual expression levels in these mutant backgrounds relative to those in the wild type.

Plant Protein Extraction and Analyses: Total Phytochrome Levels and Pfr Levels

Etiolated plants sampled for analysis of phytochrome protein levels were manipulated, grown, and red light–irradiated exactly as described above for a typical *cab2::luc* bioluminescence time course. Tissue samples (~100 mg) were collected over the *cab2::luc* expression time course at the indicated times, frozen immediately in liquid N_2, and stored in the dark at −80°C until processed further.

For the determination of total phytochrome levels, crude protein extracts were prepared as described by Somers et al. (1991), except that after centrifugation at 48,000*g*, the supernatant was recovered, and the extracts were then frozen in liquid N_2 until processed further. Extracted proteins (40 μg of total protein) were subjected to SDS-PAGE, as described previously (Somers et al., 1991).

The differential susceptibility of the Pfr and Pr forms of phytochrome to partial proteolysis in vitro (Parks et al., 1989) was used as the basis for the determination of Pfr levels over the *cab2::luc* expression time course. Crude extracts for proteolysis were prepared as above but in the absence of protease inhibitors. A portion of the preflash sample was irradiated with either saturating red or far-red light provided by an LED light source (Quantum Devices, Inc., Barneveld, WI) to generate phytochrome control samples that were predominantly in either the Pfr or Pr forms, respectively. Aliquots of the protein extracts were subjected to partial proteolysis with subtilisin (25 μg/mL added at a dilution of 1:30 [protease–extract]) for 40 min at 16°C. Proteolysis was terminated as described by Parks et al. (1996), except that phenylmethylsulfonyl fluoride was omitted from the sample buffer. Subtilisin-treated protein extracts (24 μg of total protein) were subjected to SDS-PAGE in a 4.5%/8% stacking/resolving gel.

The gel-fractionated proteins were electroblotted to nitrocellulose at 100 V for 1 hr. All blocking and antibody incubation steps were in 1 × TBST (Amersham) containing 5% dried milk. Four different primary monoclonal antibodies were used in this study. 073D (Parks et al., 1989) was used at a dilution of 1:200 and recognizes both monocot and dicot forms of the phytochrome A protein, and 1.9B5A (Daniels and Quail, 1984), the monocot phytochrome A–specific monoclonal antibody, was used at a dilution of 4 μL/3 mL. Phytochrome B was detected with a mixture of three monoclonal antibodies, B1, B7, and B8 (Somers et al., 1991), each at a dilution of 1:100. The Tu27 monoclonal antibody (Lee et al., 1990), which recognizes the most conserved site on β-tubulin (Sullivan, 1988), was used at a dilution of 1:1000. β-Tubulin levels were used as a control for protein loading and for normalizing phytochrome levels.

Secondary goat anti–mouse Ig antibody coupled to alkaline phosphatase (Tropix, Bedford, MA) was used at a 1:5000 dilution. Immunodecorated bands were detected using the Western-*Star* Chemiluminescent Detection System (Tropix), according to the manufacturer's instructions. Chemiluminescence was detected by exposure to x-ray film and was quantified using the intensified CCD camera and photon-counting image processor (Hamamatsu Photonics Systems) that was used for detection of *cab2::luc* bioluminescence but at a sensitivity between 5 and 6.

With the exception of the SDS-PAGE and immunoblot analyses, all manipulations for the determination of total phytochrome levels were performed under a dim green safelight. Similarly, all manipulations for the determination of Pfr levels were performed under an infrared safelight described previously (Anderson et al., 1994).

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