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Distinct regulation of *CAB* and *PHYB* gene expression by similar circadian clocks

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Summary

Phytochrome B (phyB) is a major phytochrome active in light-grown plants. The circadian clock controls the expression of the *PHYB* gene. We have used the luciferase reporter gene (*LUC*) to monitor the rhythmic expression of *PHYB* in photoreceptor and clock-associated mutant backgrounds. Surprisingly, we found that *PHYB* and *CAB* expression have different free-running periods, indicating that separate circadian clocks control these genes. The effects of mutations show that the clocks share common components. This suggests that they are copies of the same clock mechanism in different locations, most likely in different cell layers. Furthermore, we show that phyB is required for a negative feedback loop that strongly antagonises the expression of *PHYB*. Compared to a system with only one clock, this regulatory complexity might allow the phase of peak expression for one clock-controlled gene to alter, relative to other genes or to changing environmental conditions.

Abbreviations: Phytochrome A, phyA; Phytochrome B, phyB; firefly luciferase gene, *LUC*; chlorophyll a/b-binding protein gene, *CAB*; 12-h light:12-h dark cycle, LD or LD (12,12); far-red, FR; red light, RL; blue light, BL; white, light WL.

Keywords: *Arabidopsis*, luciferase, oscillation, phytochrome, transgenic plants.

Introduction

To optimise growth and development in 24-h day/night cycles, organisms have evolved an endogenous clock. This circadian clock is used to anticipate changes and to co-ordinate physiology and behaviour with temporal changes in the environment (reviewed in Lumsden and Millar, 1998). We use the term 'circadian clock' to denote the smallest complete timing unit (comprising an oscillator with light input and output to overt rhythms). For the clock to be useful, it must be entrained to the local day/night cycle. Light is the most important entraining signal. In plants, it is perceived and transduced by at least two classes of photoreceptor, phytochromes and cryptochromes (Devlin and Kay, 2000; Somers *et al.*, 1998a).

Phytochromes are red or far-red (FR) photoreceptors and are a major photoreceptor family in plants (reviewed in Kendrick and Kronenberg, 1994). In *Arabidopsis*, five phytochrome genes have been isolated, *PHYA–PHYE* (Clack *et al.*, 1994; Sharrock and Quail, 1989).Phytochrome B (phyB) is the most abundant phytochrome in light-grown plants; it has also been shown to control stem and petiole elongation, chloroplast development and flowering time (Reed *et al.*, 1993). phyB has also been shown to transduce red light (RL) signals to the clock, while phytochrome A (phyA) transduces RL and blue light (BL) signals at low fluence rates (Devlin and Kay, 2000; Somers *et al.*, 1998a). Following a RL treatment, phyB can be translocated to the nucleus (Kircher *et al.*, 1999; Sakamoto and Nagatani, 1996), where it has been shown to bind indirectly to the promoter region of genes such as *circadian clock associated 1* (*CCA1*) (Martinez-Garcia *et al.*, 2000). As *CCA1* is thought to be involved in the clock mechanism, its regulation by phytochromes could contribute to the entrainment of the clock.

Rhythmic signals from the clock regulate a range of processes in *Arabidopsis*, including leaf movement (Engelmann *et al.*, 1992), hypocotyl elongation (Dowson-Day and Millar, 1999), $[Ca^{+2}]_c$ (Johnson *et al.*, 1995) and stomatal opening (Somers *et al.*, 1998b). The circadian clock also regulates the expression of multiple genes involved in photosynthesis, metabolism, development and UV protection (Harmer *et al.*, 2000; Schaffer *et al.*, 2001). The most thoroughly characterised clock-regulated genes in plants are the chlorophyll a/b-binding proteins (*CAB or LHCB*) genes (Fejes and Nagy, 1998). Many regulatory genes, including *PHYA* and *PHYB*, are also regulated by the clock (Bognar *et al.*, 1999; Hall *et al.*, 2001).

A powerful tool in the dissection of circadian clocks has been genetic screening for mutants or misexpressing lines that have altered circadian periods or abolished circadian rhythms (Young and Kay, 2001). Some of the genes that affect the clock are thought not to function in the circadian oscillator, which is the minimal set of components required to produce the free-running 24-h rhythm. The *phy* mutants alter the period because the *PHY* genes are involved in light input, for example, though they share some of the properties expected of oscillator components (Bognar *et al.*, 1999). The *elf3-1* mutant causes arrhythmia selectively under constant light (Hicks *et al.*, 1996). *elf3* function is dispensible for the oscillator but is required for a negative feedback from the clock to light signalling pathways (the *zeitnehmer*) that permits rhythmicity in constant light (Covington *et al.*, 2001; Liu *et al.*, 2001; McWatters *et al.*, 2000). The *lhy* mutant (Schaffer *et al.*, 1998) and *CCA1* overexpresser (Wang and Tobin, 1998), in contrast, probably cause arrhythmic expression of *CAB* due to the functions of these genes in the oscillator mechanism (Alabadi *et al.*, 2001).

Several investigators have sought to determine whether all circadian rhythms in plants are controlled by a single clock. Simple models indicate that a single clock can control multiple overt rhythms with different phases (Wood *et al.*, 2001), but with only one period. In such systems, rhythms with different periods require different clocks; more precisely, the period is a property of the oscillator, so different periods require different oscillators. Plant rhythms with different periods have been found in several species. In tobacco, for example, the period of $[Ca^{+2}]_c$ differs from that of *CAB* expression (Sai and Johnson, 1999). In *Phaseolus vulgaris*, the periods of leaf movement and stomatal conductance differ (Hennessey and Field, 1992). The biochemical basis for such divergent periods is not clear (Millar, 1998). The underlying oscillators might have distinct biochemical mechanisms that give different periods. Alternatively, copies of one oscillator mechanism might be spatially separated, for example in different cells, with periods modified by tissue-specific factors. Any such factor must affect the oscillator if they are to alter the period, either directly or via an input pathway: factors that only affect output pathways could alter the phase, amplitude or waveform of an overt rhythm but not its period.

We address these questions by monitoring *PHYB* gene expression patterns with a luciferase reporter gene (*LUC*), in plants with modified photoreceptor and circadian clock function. *PHYB* is expressed throughout the aerial organs of *Arabidopsis*, whereas *CAB* expression is confined to mesophyll and guard cells. We identify an unexpected period difference between *CAB* and *PHYB* gene expression rhythms. However, the circadian clocks controlling *CAB* and *PHYB* expression share both photoreceptors and clock-associated genes, indicating that their biochemical mechanisms overlap considerably or are identical. We found that phyB exerts negative autoregulatory control of *PHYB* gene expression, establishing a further parallel between *PHYB* and oscillator genes.

Results

The rhythmic expression of PHYB *has a longer free running period than* CAB *expression*

We have previously shown, using a phyB promoter luciferase fusion (*PHYB:LUC*), that *PHYB* expression, in *Arabidopsis* is regulated by the circadian clock (Bognar *et al.*, 1999). We therefore characterised the rhythmic luminescence of *PHYB:LUC* plants in detail by video imaging under various light conditions, using the well-characterised *CAB:LUC* reporter lines for comparison. It is evident from the plots of luminescence in Figure 1a that the free running periods differed for *PHYB* and *CAB* expression, though the rhythms are in phase at the start of the experiment, they are approximately 4 h out of phase after 4 days in constant light. Under extended exposure to constant light the *PHYB* rhythm dampens, making comparison of the *PHYB* and *CAB* rhythms difficult after 5 days. When seedlings were imaged from the side, *PHYB* expression in the hypocotyl and roots was also rhythmic, with a very similar period, amplitude and mean level to the rhythm from the cotyledons (AH and AJM, unpublished results). *CAB* is not expressed in the roots and very weakly in the hypocotyl, so all other images were collected from above the seedling: the cotyledons and apical region with emerging primary leaves contribute essentially all of the *PHYB:LUC* signal in such images. Quantitative analysis of the free running period under constant white light (WL) revealed that expression of *PHYB* had a period approximately 1 h longer than the expression of *CAB* (Table 1). The circadian period difference was observed in multiple transgenic lines for each construct. The period difference indicates that the rhythmic expression of *PHYB* is driven by a separate oscillator to that driving *CAB* expression. We tested the accumulation patterns of *CAB* and *PHYB* RNA to confirm in *Arabidopsis* the

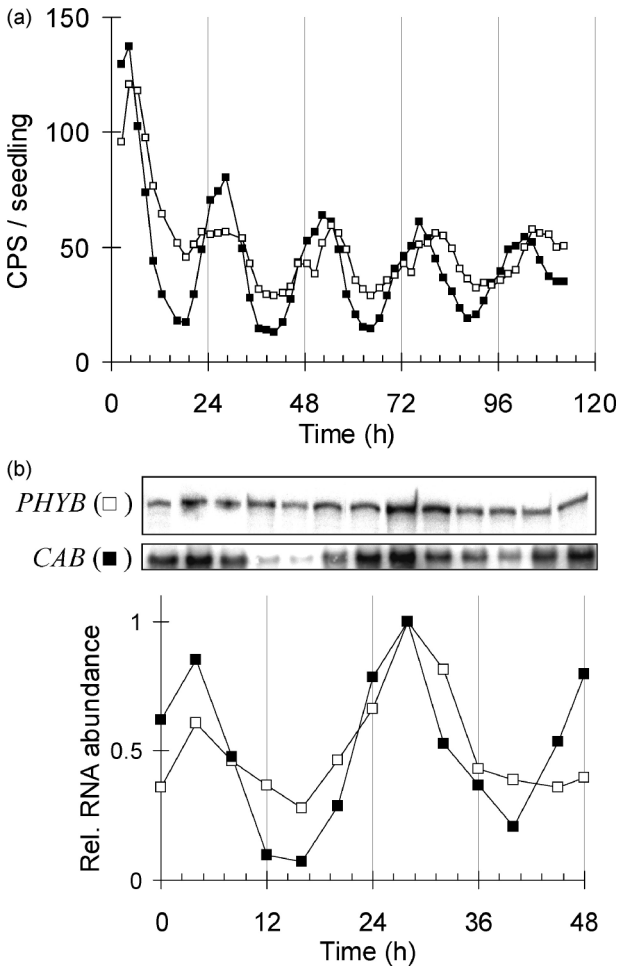

Figure 1. Circadian expression of *PHYB* and rhythmic accumulation of *PHYB* mRNA has a longer period than the circadian expression of *CAB* and accumulation of *CAB* mRNA.
(a) Groups of seedlings were grown for 7 days under 12 h light/12 h dark cycles and luminescence rhythms were assayed under constant WL. Filled squares, *CAB:LUC*, line 2CA/C. Open square, *PHYB:LUC*, line C24-19a. The data are representative of that analysed in Table 1.
(b) RNA was isolated from tissue harvested at 4-h intervals, under conditions identical to those described in (a), above. RNA gel blots were probed with a CAB coding region probe or a probe specific for *PHYB*.

rhythmic regulation of *PHYB* RNA abundance that was previously reported in tobacco (Bognar *et al.*, 1999). The period of rhythmic *CAB* mRNA accumulation was also shorter than that of *PHYB* (Figure 1b), though the difference was less clear than the reporter activity results, due to the short time course and lower time resolution.

It has been shown that the Columbia and Landsberg *erecta* accessions of Arabidopsis have slightly different free running periods for leaf movement (Swarup *et al.*, 1999). WS and C24 accessions also have different free running periods of *CAB* expression (Table 1). In both accessions the period difference between *CAB* and *PHYB* expression remains approximately 1 h (Table 1).

Table 1 The period of the *PHYB* expression rhythm is *c.* 1 h longer than the period of *CAB* expression

	Period (h)	SD	SEM	*n*
CAB:LUC				
WS-6 A	23.48	0.90	0.20	21
WS-6B	23.39	0.93	0.20	22
WS-3 A	23.21	0.79	0.15	26
PHYB:LUC				
WS-21 A	24.21	0.51	0.12	18
WS-24B	24.60	0.53	0.12	18
WS-18 A	24.90	0.92	0.22	17
CAB:LUC				
C24-1 A	25.33	0.58	0.12	23
C24-2 A	25.13	0.58	0.13	21
C24-3 A	24.97	0.77	0.17	20
2CA/C	25.45	0.23	0.05	18
PHYB:LUC				
C24-19a	26.25	0.42	0.10	18
C24-3a	26.04	0.61	0.15	17

Groups of seedlings were grown for 7 days under 12-h light/12-h dark cycles and the expression was assayed under constant WL. Period estimates were derived using the FFT-NLLS program (Plautz *et al.*, 1997) on data from the second, third and fourth days of constant light. About 95–100% of samples returned a circadian period estimate (a period in the range 15–35 h). Periods given are the variance-weighted means (period) of the estimates for *n* groups, with variance-weighted standard deviations (SD) and standard errors of the mean (SEM). Two-factor ANOVA indicated that the difference between *CAB* and *PHYB* periods was highly significant both in WS ($F=77.0$; $P<0.0001$) and in C24 ($F=32.7$; $P<0.0001$). The data shown in the table is from a single experiment; an identical period difference was observed in two exact replicates and several other experiments.

Altering light input to the clock has a similar effect on the circadian rhythms of CAB *and* PHYB *expression*

Phytochromes have been clearly identified as having an effect on the free running period of *CAB* expression. To investigate whether phytochromes have a similar effect on the circadian expression of *PHYB*, the *PHYB:LUC* marker was crossed into the phyA and phyB mutants and an *Arabidopsis PHYB* over-expressing line (ABO) (Wagner *et al.*, 1991). The circadian expression of *PHYB* was assayed under constant WL and constant RL (Figure 2). Overexpression of *PHYB* caused a RL-specific shortening of the period (approximately 1 h) of *PHYB* expression (Figure 2a), similar to that described for *CAB* expression. Under WL, the period of *PHYB* and *CAB* expression was unaffected in the ABO lines but the phase of *PHYB* expression was 2 h earlier in ABO than in the wild type (Figure 2b). The *PHYB*-specific phase change is suggestive of control by a clock that is separated from the clock regulating *CAB*, though alterations to output pathways could also be involved.

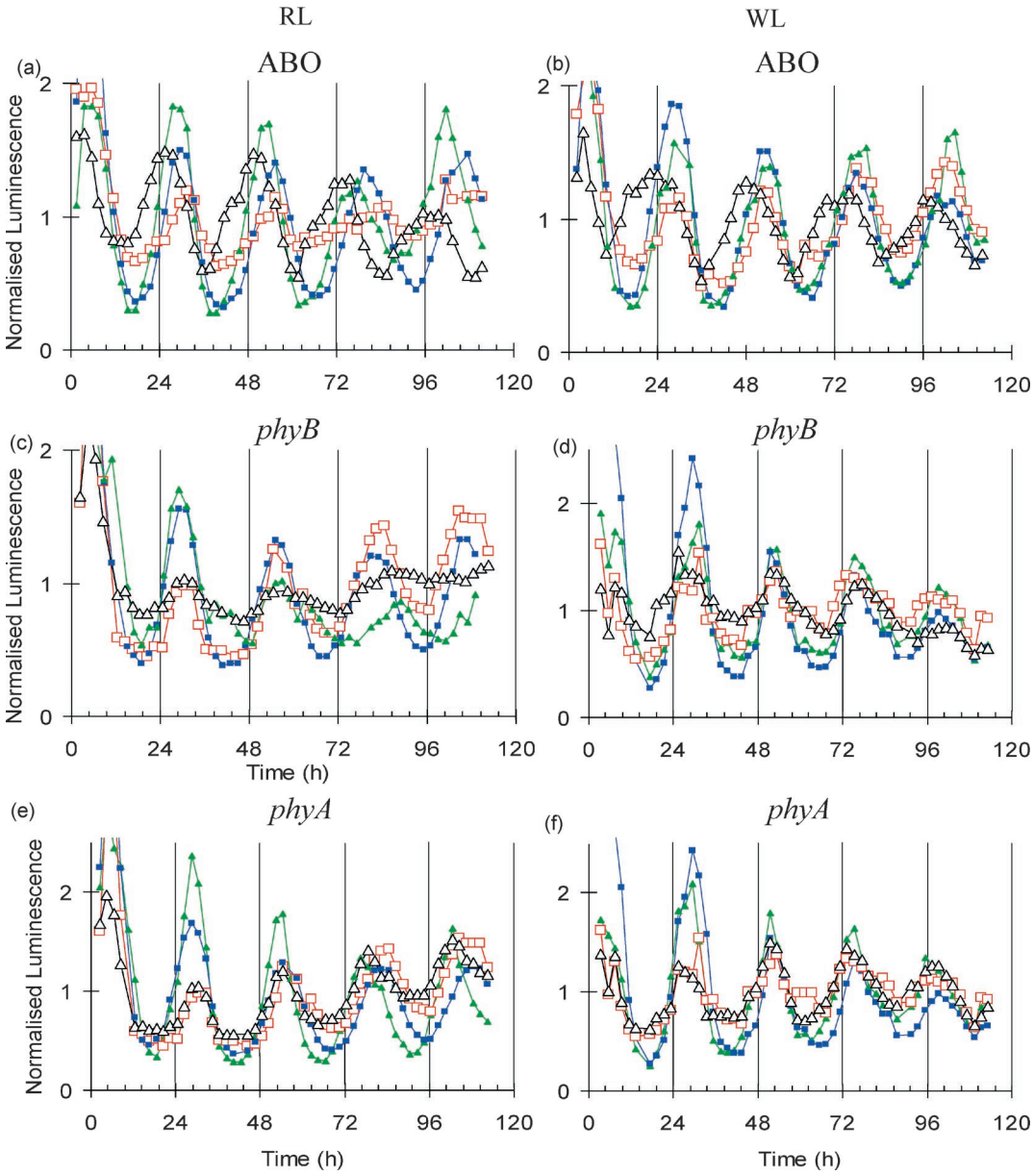

Figure 2. Altering light input to the clock affects the circadian expression of *PHYB* and *CAB* in a similar manner. Seedlings were germinated and grown for 7 days under 12 h light/12 h dark cycles. The luminescence of seedlings carrying either the *PHYB:LUC* construct (open symbols) or the *CAB:LUC* construct (filled symbols) was assayed in seedlings transferred at time 0 to constant RL (a, c, e) or to constant WL (b, d, f). In each plot, triangles represent mutant seedlings and squares represent WT seedlings.
(a, b) Overexpression of *Arabidopsis PHYB* causes a shortening of the free running period of both *PHYB* and *CAB* under RL. Under WL overexpression of *Arabidopsis PHYB* has no effect on the free running period of *CAB*, though it caused a shift in the phase of *PHYB* expression. Open triangles, *PHYB:LUC* line C24-19a crossed with ABO. Open squares, *PHYB:LUC* line C24-19a. Filled triangles, *CAB:LUC* line 2CA/C crossed with ABO. Filled squares, *CAB:LUC* line 2CA/C.
(c, d) The absence of phyB lengthens the free-running period of *CAB* and *PHYB* under RL. Open triangles, *PHYB:LUC* line WS-21a crossed with phyB-464–19. Open squares, *PHYB:LUC* line WS-21a. Filled triangles, *CAB:LUC* line 2CA/C crossed with phyB-1. Filled squares, *CAB:LUC* line 2CA/C.
(e, f) The absence of phyA has no effect on the free running period of *CAB* and *PHYB* under RL or WL. Open triangles, *PHYB:LUC* line WS-21a crossed with phyA-410. Open squares, *PHYB:LUC* line WS 21a. Filled triangles, *CAB:LUC* line 2CA/C crossed with phyA-201. Filled squares, *CAB:LUC* line 2CA/C.

The period of *CAB* expression lengthened and the amplitude of the oscillation decreased in the phyB mutant under RL; a similar 2-h period lengthening and decreased amplitude was observed for the *PHYB* rhythm (Figure 2c). In WL, the phyB mutation had no effect on the period of either marker (Figure 2d). The wild-type period of both phyB and ABO under WL suggests that BL receptors modulate the circadian expression of *PHYB*, as they do for *CAB* (Devlin and Kay, 2000; Millar *et al.*, 1995; Somers *et al.*, 1998a). At the RL fluence rate used in this experiment

($30\ \mu mol\ m^{-2}\ s^{-1}$), a lack of phyA was reported to have little effect on the period of *CAB* expression (Somers *et al.*, 1998a). We found no period-lengthening effect of the phyA mutation upon either the *CAB* or *PHYB* expression rhythms at this fluence of RL (Figure 2e) or in WL (Figure 2f). Taken together, these results indicate that the circadian clocks driving *PHYB* and *CAB* expression share very similar light input pathways.

PhyB negatively regulates PHYB *expression*

The data in Figure 2 were normalised to the mean, in order to facilitate the comparison of rhythmic waveforms. When the mean expression levels were compared, the absence of phyB was found to cause a 10-fold increase in mean *PHYB:LUC* activity under WL and RL (Figure 3a), indicating that phyB negatively regulates *PHYB* expression. Mean *PHYB:LUC* activity is also about fivefold higher in wild-type plants during dark adaptation than under RL (Figure 3a), as previously reported (Bognar *et al.*, 1999; Goosey *et al.*, 1997; Reed *et al.*, 1993).

Active phyB is present in the wild type for the first few hours of darkness, so there was a clear difference between wild-type and phyB over the first 12 h (Figure 3b). After 24 h, however, the down-regulation of *PHYB* expression by phyB is relieved, even in the wild type (Figure 3b). The mean level of *PHYB:LUC* activity in prolonged darkness was therefore almost as high in the wild type as in the phyB mutant (Figure 3a). An early increase in *PHYB:LUC* activity could be provoked in wild-type plants by an end-of-day FR light treatment. This converts light-stable phytochromes from the active (Pfr) to inactive (Pr) form, and causes a two- to three fold increase in *PHYB:LUC* activity within 1 h (Figure 3c). We observed the FR-induction of *PHYB:LUC* activity in multiple independent wild-type lines and also in the phyA mutant; little induction was observed in the phyB mutant, because the starting level was already greatly increased (Figure 3c). Interestingly, the peak phase for both *CAB* and *PHYB* expression was consistently advanced by 2–3 h in the phyB mutant, relative to wild type (Figure 3b).

The absence of phyA was found to increase *PHYB* expression in RL and WL, though to a lesser extent than the lack of phyB (Figure 3a). Active phytochromes A and B therefore feed back to inhibit *PHYB* expression. Overexpression of *PHYB* did not reduce the *PHYB* expression below the wild-type level, suggesting that the negative feedback circuit is saturated in the wild type. The biological activity of the over-expressed protein is confirmed by the increased level of *CAB* expression in darkness (Figure 3a) and the altered period of *PHYB* (Figure 2a). This negative autoregulation of *PHYB* expression is exactly opposite to the phytochrome-mediated activation of *CAB* expression: in the dark and in phyB mutants, *CAB* expression is reduced and in the light *CAB* expression increases (Figure 3a).

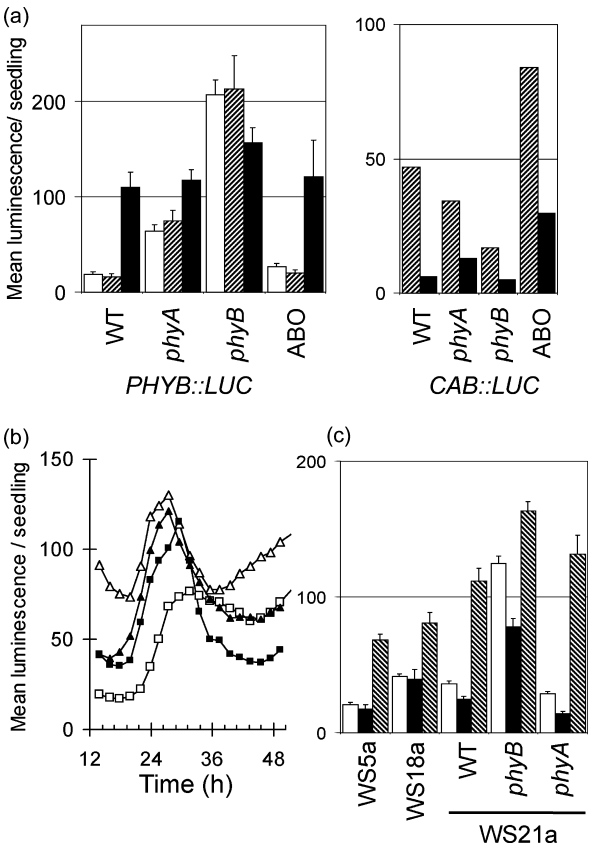

Figure 3. phyB reduces *PHYB* expression level in light-grown seedlings.
(a) *PHYB:LUC* activity levels in light and darkness. The mean luminescence per seedling from the experiments shown in Figure 2 is plotted, for seedlings transferred to WL (open column) or RL (hatched column), and from equivalent experiments with a transfer to darkness at time 12 (filled column). The graphs show the average luminescence of six groups of plants over 5 days under constant conditions; error bars represent one SEM; WT, wild type.
(b) *PHYB:LUC* activity pattern in darkness. Groups of seedlings were entrained as in Figure 2, then imaged after transfer to constant darkness at 12 h (where 0 is lights-on, as in Figure 2). Open triangles, *PHYB:LUC* line WS-21a crossed with phyB-464–19. Open squares, *PHYB:LUC* line WS-21a. Filled triangles, *CAB:LUC* line 2CA/C crossed with phyB-1. Filled squares, *CAB:LUC* line 2CA/C.
(c) Induction of *PHYB:LUC* by FR light treatment. Groups of seedlings were entrained and imaged as in Figure 2. Luminescence per seedling is plotted at the end of the day (time 12 h, open columns) and 1 h following transfer either to darkness (filled columns) or to FR light (hatched columns). The strain designations refer to independently transformed *PHYB:LUC* lines in the wild-type WS background; the transgene from line WS21a was introduced into phyA and phyB backgrounds by crossing from the wild type (WT). The graphs show the average luminescence levels of four groups of seedlings; error bars represent one SEM.

Over-expression of CCA1 *or* LHY *and the* elf3 *mutation cause arrhythmia in* PHYB *expression*

To investigate whether the two circadian clocks contained common components the *PHYB:LUC* marker was introduced into the *elf3-1* and *lhy* mutants, and the *CCA1* over-expressing line. These mutants cause arrhythmic *CAB* expression in constant light (Hicks *et al.*, 1996; Schaffer

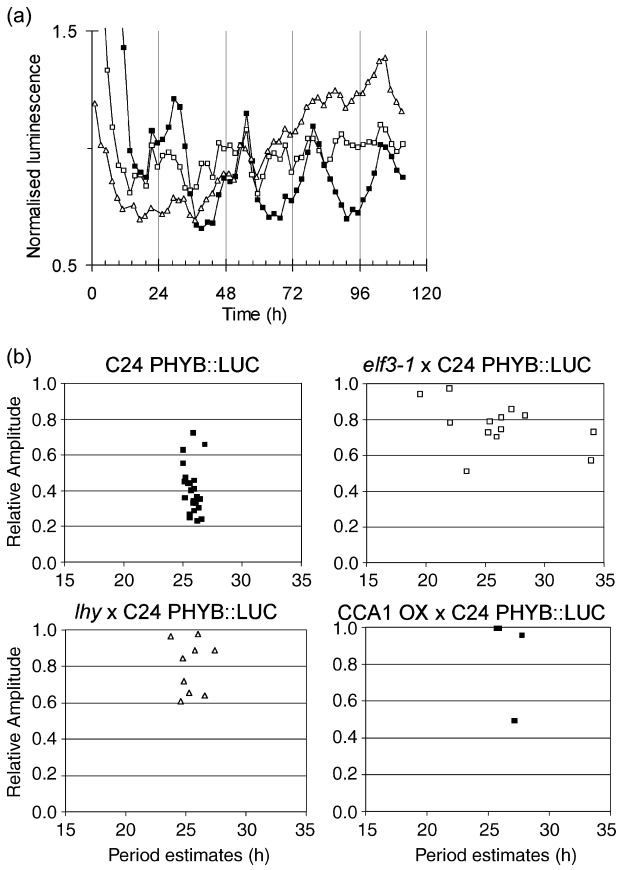

Figure 4. *PHYB* expression is arrhythmic in elf3, lhy and CCA1 over-expressing backgrounds. Seedlings were germinated and grown for 7 days under 12 h light/12 h dark cycles. Expression from the *PHYB* promoter was assayed under constant WL.

(a) The expression of *PHYB* is arrhythmic in the *elf3-1* and the *lhy* mutants. Filled squares, *PHYB:LUC* line C24-19a. Open squares *PHYB:LUC* line C24-19a crossed with *elf3-1*. Open triangles, *PHYB:LUC* line C24-19a crossed with *lhy*.

(b) The scatter plots represent rhythmic periods between 15 and 35 h estimated by FFT-NLLS analysis (Dowson-Day and Millar, 1999) and plotted against the relative amplitude error (RAE). The RAE is a measure of rhythm robustness. Tight clustering with a low RAE indicates a robust rhythm. The periods of *PHYB* expression in both lhy and elf3-1 show little clustering and high relative amplitude errors indicating weak rhythms or noise.

Table 2 The circadian expression of *PHYB* is arrhythmic in *elf3-1*, lhy and CCA1 OX. FFT-NLLS analysis of the circadian expression of *PHYB* in WT, *elf3-1*, lhy and CCA1 OX seedlings

	Period (h)	SD	SEM	*n*	Circadian periods (15–35 h)
PHYB:LUC					
C24-19a	25.79	0.43	0.09	24	24
C24-19a × *elf3-1*	24.44	3.25	0.9	24	13
C24-19a × CCA1-OX	26.82	0.74	0.37	18	4
C24-19a × *lhy*	25.08	0.89	0.3	24	9

Analysis was performed on groups of 30–40 seedlings, n = the number of groups analysed.

et al., 1998; Wang and Tobin, 1998). The expression of *PHYB* was then assayed under constant WL. In all three mutants, the expression of *PHYB* appeared to be arrhythmic (Figure 4a; data not shown). The period analysis software failed to detect circadian rhythms in a high percentage of samples and the minority of rhythms detected had high RAE values and were scattered across the circadian range (15–35 h) as is typical of arrhythmic mutants (Figure 4b; Table 2). In the *CCA1* over-expression background, a similar arrhythmicity was observed for both reporters (Figure 4b; Table 2). The arrhythmicity of *PHYB:LUC* suggests that the CCA1, LHY and ELF3 proteins function similarly in the circadian regulation of both *CAB* and *PHYB*.

Discussion

We used transgenic *Arabidopsis* carrying the *PHYB:LUC* and *CAB:LUC* reporters to compare the circadian rhythms of *PHYB* and *CAB* expression (Figure 1). The free-running period of *PHYB* expression was approximately 1 h, longer than that of *CAB* expression, in multiple transgenic lines and two genetic backgrounds (Table 1). This indicates that the *PHYB* and *CAB* expression rhythms are regulated by separate clocks. Previous reports document differences in the period of plant circadian rhythms (Hennessey and Field, 1992; Sai and Johnson, 1999), though they did not address the molecular mechanisms of the underlying clocks. We therefore tested the *PHYB* and *CAB* expression rhythms in *phy* mutant and *PHYB* over-expression lines, under white and RL. The periods of both rhythms were altered in parallel by manipulating the levels of active phytochrome (Figure 2). The period effects on both rhythms showed the same dependence on the ambient light conditions and on the form of phytochrome (Figure 2). These data show that the same light input components regulate the period of both clocks.

Comparing the levels of *PHYB* expression in the mutant lines, our data show that wild-type phyB reduces *PHYB* expression in constant RL or WL, by approximately 10-fold compared to its level in a *phyB* mutant (Figure 3a). This is broadly in agreement with a previous study based upon a *PHYB:*GUS fusion construct (Somers and Quail, 1995a). The effect of the *PHYB* mutation on GUS activity was less than we observed, possibly due to the differences in the reporter genes. phyA negatively regulates *PHYB* expression levels approximately threefold in constant RL or WL, illustrating the complex interaction among phytochrome species (Figure 3a). The negative feedback control of *PHYB* gene expression level provides a further similarity between *PHYB* and circadian oscillator components. Its effect on rhythms *in vivo* remains to be tested and is not easy to predict. The rhythmicity of the *phyA, phyB, cry1 and cry2* mutant (Yanovsky *et al.*, 2000) suggests that photoreceptor rhythms are more likely to fine-tune overt rhythms or light

responses than to participate centrally in the oscillator mechanism. The phase advances that we observed for *PHYB* expression in the *PHYB* over-expressing background (Figure 2b), and for both *CAB* and *PHYB* in the *phyB* mutant (Figure 3b), are the type of minor changes in rhythmicity that might be due to alterations in the negative feedback.

We tested the expression of *PHYB* in *elf3-1* mutants and in *LHY* and *CCA1* overexpressors, in order to determine whether the *PHYB* rhythm requires these components of the *zeitnehmer* and oscillator that control *CAB* expression. *PHYB* expression was arrhythmic under constant light in these backgrounds, as is *CAB* expression (Figure 4). We conclude that several components of the light input pathway, *zeitnehmer* and oscillator function similarly in the circadian clocks that regulate *CAB* and *PHYB* expression. We found no evidence of a qualitative distinction between the molecular mechanisms of the two clocks: their mechanisms are very similar, if not identical.

It is formally possible that a single cell could maintain two spatially separated pools of clock proteins, supporting rhythms with two periods in the same cell; there are no precedents or known mechanisms to support this notion. We, therefore, propose that copies of the clock mechanism in separate cells are modified in a tissue-specific manner, such that the clocks in different cell types are independent and have different free running periods. *CAB* and *PHYB* are expressed in different spatial domains. Numerous observations report that *CAB* expression is restricted to leaf mesophyll and guard cells, in light-grown seedlings (Kretsch *et al.*, 1995), whereas *PHYB* is expressed in both leaf epidermis and mesophyll, and more widely throughout the plant (Bognar *et al.*, 1999; Somers and Quail, 1995b). The seedlings in this paper were imaged from above: almost all the luminescence detected derives from the cotyledons and apical region. Within the aerial tissues, luminescence from the epidermis is detected preferentially, because signal from the mesophyll is diminished by passage through the intervening cell layers (Wood *et al.*, 2001). The longer period of *PHYB* expression in our assays is therefore most likely to represent the rhythmic expression in the epidermis. *PHYB:LUC* expression in the mesophyll will certainly contribute to the signal that we record. If this shares the circadian period of *CAB* expression in the same cells, the measured period difference in total luminescence of approximately 1 h will underestimate the true period difference between cell layers. Consistent with this hypothesis, the period of chalcone synthase gene expression, which is strongly enriched in the epidermis, is approximately 1.5 h longer than the period of *CAB* expression under constant light (Thain *et al.*, 2002). The construction of chimeric plants that express *PHYB:LUC* in a restricted range of cells would be required to measure its rhythmic period specifically in the mesophyll or epidermis.

The difference in periods under constant conditions implies that the clocks controlling *CAB* and *PHYB* are functionally independent. We have previously shown that areas of a single leaf do not communicate timing information within a cell layer (Thain *et al.*, 2000); our current results indicate that clocks in neighbouring cell layers are also independent. Our conclusion further implies that tissue-specific factors can modify the period of the circadian clock; the identity of these factors is unknown. Variation in photoreceptor concentration among cell types could produce such period differences. Altering photoreceptor activity is known to affect circadian period (Figure 2; Somers *et al.*, 1998a) and there is some evidence that photoreceptor gene expression varies among cell types (Somers and Quail, 1995b). Organisms with many independent clocks might gain adaptive advantages from the greater flexibility in the phase of rhythms relative to the light/dark cycle or to other circadian rhythms (Pittendrigh and Daan, 1976; Roenneberg and Mittag, 1996), compared to a system with a single circadian pacemaker. This potential advantage can be realised only if the individual clocks vary in some way. We have shown that the circadian clocks of *Arabidopsis* are heterogeneous and independent, potentially optimising plant responses under changing photoperiods.

Experimental procedures

Plant materials and probes

The *PHYB:LUC* construct used to create independent transformants in the WS and C24 backgrounds has been described (Bognar *et al.*, 1999). The *CAB:LUC* line 2CA/C has been described (Millar *et al.*, 1992b). The *CAB:LUC+* construct used to create the independent transformants in the WS and C24 backgrounds contained a transgene in which the *LUC* gene was replaced with the *LUC+* gene (Promega, Madison, WI, USA). *CAB:LUC* lines in the phyA-201, phyB-1 and PHYB over-expressing backgrounds have been described (Anderson *et al.*, 1997), as have *CAB:LUC* lines in *elf3-1* (Hicks *et al.*, 1996) and *lhy* (Schaffer *et al.*, 1998). The *CAB:LUC* reporter from line 2CA/C was combined with *CCA1* over-expression line 038 (Wang and Tobin, 1998) by similar methods. *PHYB:LUC* line WS-21a was combined with the phyA-410 and phyB-464–19 (Reed *et al.*, 1993) mutations in the WS background by crossing and selection essentially as described (Anderson *et al.*, 1997). *PHYB:LUC* line C24-19a was similarly combined with the *PHYB* over-expressing line, *elf3-1*, *lhy* and *CCA1* over-expression line 038. Probes and methods used for the RNA analysis in Figure 1b were as described (Bognar *et al.*, 1999).

Growth conditions

Arabidopsis plants were grown in sterile conditions as described (Millar *et al.*, 1992a). Seeds were stratified at 4°C for 4 days, then germinated and entrained for 7 days in a 12-h light (white fluorescent bulbs 80 μmol m^{-2} s^{-1}) or 12-h dark cycle at 22°C. Seedlings were imaged under constant WL (white fluorescent bulbs, 40 μmol m^{-2} s^{-1}) or constant RL (Growlux bulbs covered in Fiery Red filter (Lee Filters, Andover, UK), 30 μmol m^{-2} s^{-1}). For the FR

light treatment, a 180 LED array of KL450–730D LEDs (Shinkoh electronics, Tokyo, Japan) was used, giving approximately 80 μmol $m^{-2} s^{-1}$.

Luminescence measurement and period analysis

Seedlings were imaged in clusters of 20–30 plants, plastic transparent collars were placed around seedlings with long hypocotyls to prevent spreading during the experiment. Luciferase luminescence was measured using an intensified CCD camera (Hamamatsu VIM, Hamamatsu City, Japan) or a cryogenically cooled CCD camera (Roper Scientific). The images were processed using Metamorph software (Universal Imaging Corp.). Data analysis was performed using the landA Excel macro suite (http://www.scripps.edu/cb/kay). Period estimates were obtained using the Fast Fourier Transform – Non-Linear Least Squares program (FFT-NLLS) as described (Dowson-Day and Millar, 1999; Plautz *et al*., 1997).

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