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Circadian Clock-Regulated Expression of Phytochrome and Cryptochrome Genes in Arabidopsis

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Many physiological and biochemical processes in plants exhibit endogenous rhythms with a period of about 24 h. Endogenous oscillators called circadian clocks regulate these rhythms. The circadian clocks are synchronized to the periodic environmental changes (e.g. day/night cycles) by specific stimuli; among these, the most important is the light. Photoreceptors, phytochromes, and cryptochromes are involved in setting the clock by transducing the light signal to the central oscillator. In this work, we analyzed the spatial, temporal, and long-term light-regulated expression patterns of the Arabidopsis phytochrome (PHYA to PHYE) and cryptochrome (CRY1 and CRY2) promoters fused to the luciferase (LUC* ) reporter gene. The results revealed new details of the tissue-specific expression and light regulation of the PHYC and CRY1 and 2 promoters. More importantly, the data obtained demonstrate that the activities of the promoter:LUC* constructs, with the exception of PHYC:LUC*, display circadian oscillations under constant conditions. In addition, it is shown by measuring the mRNA abundance of PHYA and CRY genes under constant light conditions that the circadian control is also maintained at the level of mRNA accumulation. These observations indicate that the plant circadian clock controls the expression of these photoreceptors, revealing the formation of a new regulatory loop that could modulate gating and resetting of the circadian clock.

The periodic succession of days and nights is an externally recurring environmental factor ever since life has appeared on the Earth. It is postulated that organisms possessing the ability to adapt to the predictable changes of the environment have an evolutionary advantage and that this benefit has promoted the development of timekeeping mechanisms (endogenous clocks). The biological clocks that generate and maintain oscillations of many physiological and molecular processes with a period length close to 24 h are also referred to as circadian clocks. Circadian rhythms persist under constant conditions; however, to function reliably and to be useful for the organisms, the clocks must operate in harmony with the periodic changes of the outer environment. To achieve this synchrony, the circadian clock is reset to the local time by specific stimuli perceived at dawn and dusk. The most important entraining factors are light and temperature. Light signals are perceived and transduced to the central oscillator via specialized photoreceptors. In plants, the photoreceptor phytochrome and cryptochrome have been shown to be involved in this process (Somers et al., 1998; Devlin and Kay, 2000).

Phytochromes are chromoproteins that contain a covalently linked linear tetrapyrrole chromophore per molecule and exist as homodimers. These photoreceptor molecules absorb red and far-red light, which activates or inactivates them, respectively (Quail et al., 1995). In higher plants, small multigenic families encode these molecules. In Arabidopsis, five genes (PHYA–E) have been isolated (Sharrock and Quail, 1989; Clack et al., 1994). PHYA is a photolabile molecule degrading rapidly upon exposure to light. It is the dominant phytochrome in etiolated seedlings and it mediates responses to very low fluences of red and far-red light. PHYB, C, D, and E are relatively photostable molecules; in green seedlings, PHYB is the dominant phytochrome photoreceptor. They mediate responses to low and high fluences of red light (for review, see Furuya and Schäfer, 1996; Casal et al., 1998; Neff et al., 2000). Recently, it was shown that PHYA and PHYB translocate to the nucleus in a light-dependent manner (Kircher et al., 1999; Yamaguchi et al., 1999). It was also suggested that the regulated nuclear import of these receptors could be a key element of the phytochrome signal transduction pathway (Nag and Schäfer, 2000a).

Plant cryptochromes are FAD and pterin-containing chromoproteins showing significant homology to DNA photolyases, but lacking photolyase activity. Cryptochromes absorb in the blue region of the spectrum. To date, two members of the cryptochrome family, CRY1 and 2, have been identified in Arabidopsis (Ahmad and Cashmore, 1993; Lin et al., 1998). The CRY2 protein shows rapid blue light-induced degradation and functions primarily at low light intensities (Lin et al., 1998). The CRY1 protein is

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relatively stable in light and mediates responses to higher fluences of blue light (Lin et al., 1998). More recently, cryptochrome photoreceptors were also identified in animals. In Drosophila melanogaster, input light signals absorbed by the CRY photoreceptors promote the degradation of the TIMELESS protein, a key element of the fly’s circadian oscillator, thereby resetting the clock (Ceriani et al., 1999; Emery et al., 2000). Although it is unclear whether the CRY protein is a functional input receptor to the mammalian clock, it was proven that it is an indispensable part of the central oscillator in mammals (van der Horst et al., 1999).

According to the simplest model of the circadian system, the central oscillator generates an oscillation with a period of approximately 24 h, based on negative feedback loops formed by the clock genes and proteins, and it regulates the expression of genes through the output pathway. On the other side of the system, light signals absorbed by photoreceptors reach the central oscillator through the input pathway and synchronize its phase to the actual periodic environmental changes. In this model, there is a one-way relationship between the input receptors and the oscillator without any feedback mechanisms. However, it has been shown that the oscillator controls the expression of cryptochrome receptors in D. melanogaster and in mouse (Mus musculus; Glossop et al., 1999; Shearman et al., 2000). We have demonstrated previously that the Arabidopsis circadian clock regulates the expression of the PHYB photoreceptor (Kozma-Bognár et al., 1999). Very recently, Harmer and coworkers (Harmer et al., 2000) demonstrated that the mRNA levels of the Arabidopsis CRY1 and CRY2 genes oscillate with a circadian rhythm under constant light (LL) conditions.

In this work, we performed a detailed, comparative analysis of the expression patterns of all phytochrome and cryptochrome genes in Arabidopsis with respect to their circadian regulation. Utilizing the luciferase reporter system, we demonstrate that the circadian clock controls the promoter activity of all CRY and PHY genes except for that of PHYC. We also show that the circadian modulation of the promoter activities is reflected at the level of mRNA accumulation. Regarding the PHYC gene, we demonstrate that the circadian clock regulates only the accumulation of PHYC mRNA. Moreover, by using the luminescent reporters, we also present in vivo data on tissue specificity and light-regulated expression of all PHY and CRY genes.

RESULTS

Spatial Expression Pattern of the Luminescent Reporter Constructs

Transgenic seedlings expressing the various promoter::luciferase constructs were grown in light-dark cycles for 1 week. The seedlings were sprayed with 5 mM luciferin solution, and the bioluminescence patterns were characterized by in vivo imaging. The expression patterns of the PHYA::LUC<sup>+</sup> and CRY2::LUC<sup>+</sup> chimeric genes were nearly identical: A high level of luciferase activity was measured in the shoot meristems and root tips, and less but significant activity was found in the cotyledons, hypocotyls, and roots (Fig. 1, B and H). CAB2::LUC<sup>+</sup> and CRY1::LUC<sup>+</sup> were actively transcribed in the aerial tissues (in cotyledons and leaf primordia), but no activity was detected in the roots (Fig. 1, A and G). PHYB::LUC<sup>+</sup> was luminescent in all tissues with highest activity in the cells of the shoot meristem and root tips (Fig. 1C). The expression pattern of PHYD::LUC<sup>+</sup> and PHYE::LUC<sup>+</sup> was similar to that of PHYB::LUC<sup>+</sup>, but displayed relatively lower expression in the shoot meristem (Fig. 1, E and F). PHYC::LUC<sup>+</sup> was active mainly in the cotyledons and root tips, but lower expression was detected in the leaf primordia and in the root (Fig. 1D). The data presented here for the organ-specific expression of the PHYA, PHYB, PHYD, and PHYE genes are consistent with earlier reports based on β-glucuronidase (GUS) reporter fusions (Somers and Quail, 1995; Goosey et al., 1997) and mRNA accumulation (Clack et al., 1994). Moreover, they confirm the results of mRNA analysis (Ahmad and Cashmore, 1993; Lin et al., 1998) and extend our knowledge concerning the organ- or tissue-specific expression of CRY1-2 and PHYC genes.

Diurnal and Circadian Regulation of Phytochrome and Cryptochrome Promoter Activity

Transgenic seedlings carrying the various promoter::luciferase chimeric genes, including the CAB2::LUC<sup>+</sup> control, were grown under 12-h-light/12-h-dark photoperiods for 1 week and were imaged under the same conditions. All of these plants showed diurnal rhythms with activity peaking during the light phase, although with there were differences regarding amplitudes and phases (Fig. 2). The luminescence rhythm of PHYA::LUC<sup>+</sup> displayed a biphasic curve. The first peak appeared just after the lights-on signal and was followed by a second peak occurring a few hours before the lights-off signal (Fig. 2A). The amplitude of the oscillation of PHYB::LUC<sup>+</sup> activity was very similar to that of PHYA::LUC<sup>+</sup>; however, its expression peaked earlier at about 4 h after the lights were on (Fig. 2A). The activity of PHYC::LUC<sup>+</sup> was again higher during the light phase and lower in dark phase, but it showed a low amplitude rhythm. It peaked about 2 h before the lights were turned off and promptly decreased to a lower, flattened level in the dark (Fig. 2B). The diurnal rhythms of PHYD::LUC<sup>+</sup> and PHYE::LUC<sup>+</sup> were quite similar: They showed the same phase of maximal expression (4–6 h after the lights were on) and had a relatively low amplitude (Fig. 2C). The expres-
Circadian Regulation of AtPHYA–E and AtCRY1-2 Gene Expression

The expression of CRY1::LUC\(^+\) and CAB2::LUC\(^+\) had the same phase and similar amplitude (Fig. 2, D and B). In contrast, the CRY2::LUC\(^+\) rhythm showed a much lower amplitude and a late phase, with maximal expression around the end of the light phase (Fig. 2D). In all cases, except for PHYC::LUC\(^+\), the observed diurnal rhythms anticipated the lights-on and lights-off signals, suggesting a role for the circadian clock in the regulation of the expression of these genes.

The most reliable diagnostic feature of circadian rhythms is that they persist under constant conditions. Therefore, we measured the luminescence of the same transgenic seedlings entrained as described above, in LL (Fig. 3) and constant dark (DD; Fig. 4).

In LL, most of the chimeric genes showed rhythmic
expression. These data indicate that the circadian clock controls the expression of these genes. However, as compared with light/dark conditions (LD), we detected minor changes in the phase of the peaks (1–2 h) in LL. For example, in LL, PHYA and PHYB::LUC reached their maximum activities later (Fig. 3A versus Fig. 2A), whereas the peak luminescence of PHYD and PHYE::LUC was shifted to an earlier time (Fig. 3C versus Fig. 2C) than in LD. As an exception, the expression of PHYC::LUC showed a very weak rhythmicity, if any, in LL (Fig. 3B). Its pattern is characterized by a much-reduced amplitude and a 12-h phase-shift (from Zeitgeber time [ZT] 12 to ZT 24).

In DD, many of the circadian processes dampen rapidly in plants. For example, the rhythmic expression of CAB2::LUC dampered to a low level in DD (Fig. 4B). The opposite effect was observed for the PHYA::LUC and CRY2::LUC constructs. The activity of these transgenes dampened to a high level after a rapid initial increase during the first subjective day (Fig. 4, A and D). The luminescence of PHYB::LUC also showed this initial increase (Fig. 4A); however, its rhythm did not dampen rapidly and exhibited about 2-fold lower amplitude compared with the corresponding LL data (Fig. 3A). The amplitude of the CRY1::LUC rhythm decreased day by day during the measurement (Fig. 4D). The extended dark period did not significantly reduce the amplitude of the PHYD,E::LUC rhythms, as shown in Figure 4C. The expression level of PHYC::LUC decreased continuously in the dark and had an extremely low amplitude with two maxima at ZT 24 and ZT 48 (Fig. 4B), similar to LL (Fig. 3B).

**Long-Term Regulation of the Mean Level of Phytochrome and Cryptochrome Promoter Activities by Light**

Seedlings carrying the various promoter::luciferase reporter constructs were grown and entrained in LD cycles for 1 week and were subsequently transferred to LL or DD. Luminescence of individual seedlings was measured using the TopCount luminometer (Packard Instruments, Meriden, CT) for 4 d as described in Figure 5. The average of the counts col-

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Figure 2. Diurnal regulation of phytochrome and cryptochrome gene expression in Arabidopsis seedlings. Seedlings were grown under 12-h-light/12-h-dark cycles for 1 week, and were then imaged under the same conditions. A, PHYA::LUC (○); PHYB::LUC (▲); B, PHYC::LUC (○); CAB2::LUC (△); C, PHYD::LUC (○); PHYE::LUC (▲); D, CRY1::LUC (○), CRY2::LUC (▲). White box on time axis, Light interval; black box, dark interval.

Figure 3. Circadian regulation of phytochrome and cryptochrome gene expression in LL. Seedlings were grown and entrained as in Figure 2, but were imaged after transfer to LL. A, PHYA::LUC (○); PHYB::LUC (▲); B, PHYC::LUC (○); CAB2::LUC (△); C, PHYD::LUC (○); PHYE::LUC (▲); D, CRY1::LUC (○), CRY2::LUC (▲). White box on time axis, Light interval; striped box, subjective dark interval.
lected during the whole measurement from seedlings with the same transgene was calculated and is referred to as the mean expression level (Fig. 5). The expression levels of the PHYA,B::LUC/CRY2::LUC constructs in the dark were 1.5- to 2.5-fold higher than in the light; thus, the activity of these promoters is down-regulated by light. In contrast, the luminescence of PHYC::LUC, CAB2::LUC, and CRY1::LUC was 2- to 3-fold higher in the light than in the dark. The activity of the PHYD,E::LUC constructs showed very weak light dependence because their expression level was only 1.2- to 1.3-fold higher in the light.

Circadian Accumulation of the Phytochrome and Cryptochrome mRNA Molecules

Wild-type Arabidopsis seeds (Wassilewskija [WS] ecotype) were germinated and grown on sterile Murashige and Skoog medium under 12-h-light/12-h-dark cycles for 1 week and were then transferred to LL. Total RNA was isolated from samples harvested in 4-h intervals. The abundance of phytochrome and cryptochrome mRNA molecules was determined as described in “Materials and Methods.”

In all cases, the mRNA levels of the various PHY and CRY genes displayed clear circadian oscillations (Fig. 6). In general, the phases of peaks for mRNA levels were the same or 2 to 4 h earlier as compared with the corresponding luminescence data (Fig. 3). Moreover, rhythms in mRNA levels of the PHYA,B (Fig. 6A) and of the CRY1,2 (Fig. 6D) genes had amplitudes similar to those of the corresponding luminescence rhythms (Fig. 3, A and D). These data suggest that the circadian rhythms originating from the clock-regulated promoter activity of these genes exist, without significant modifications, at the level of the mRNA accumulation. However, the oscillation of the PHYD and PHYE mRNA levels exhibited significantly higher (3- to 4-fold) amplitudes (Fig. 6C) than the rhythms of their promoter activity (Fig. 3C), indicating that the circadian clock has an additional effect on the mRNA synthesis/stability of these genes. The luminescence of the PHYC::LUC construct showed very weak rhythm with extremely low amplitude in LL (Fig. 3B). To our surprise, however, the PHYC mRNA level displayed clear circadian oscillations with an amplitude comparable with that of the PHYD,E mRNA levels (Fig. 6B). This fact indicates that the circadian regulation of the PHYC gene

Figure 4. Circadian regulation of phytochrome and cryptochrome gene expression in DD. Seedlings were grown and entrained as in Figure 2 and 3, but were imaged after transfer to DD. A, PHYA::LUC (○), PHYB::LUC (▲); B, PHYC::LUC (○), CAB2::LUC (▲); C, PHYD::LUC (○), PHYE::LUC (▲); D, CRY1::LUC (○), CRY2::LUC (▲). White box on time axis, Light interval; black box, dark interval; gray box, subjective light intervals.

Figure 5. Mean expression levels of the various reporter constructs in 1-week-old Arabidopsis seedlings under extended LL (white columns) or DD (black columns) conditions. Seedlings were grown and entrained as in Figure 2, and were then transferred to LL or DD conditions. Luminescence was measured in 1- to 2-h intervals for 4 d starting from ZT 24 in a TopCount luminometer. The experiment included 24 individual seedlings from each of three to four independent transgenic lines for each reporter construct. The average of counts collected during the entire measurement from seedlings carrying the same transgene was calculated and is presented in Figure 5 as the mean expression level of that construct under the conditions specified. To accommodate the large differences in expression level between the constructs, the y axis was drawn with two different scales. Note that luminescence activities presented on this figure were not calculated from graph data presented in Figures 3 and 4.
expression is exerted at the level of mRNA accumulation rather than at level of the promoter activity.

DISCUSSION

Tissue-Specific Expression

To study spatial and temporal regulation of Arabidopsis PHY and CRY genes at the level of promoter activity, we constructed a series of PHY and CRY promoter::luciferase chimeric genes and regenerated a large number of transgenic Arabidopsis plants expressing these reporters. Tissue- and organ-specific expression of the transgenes was determined at the same developmental stage (LD-grown, 7-d-old seedlings; Fig. 1) when time course measurements for circadian rhythmicity started. Spatial expression patterns of the PHYA, PHYB, PHYD, and PHYE promoter::luciferase transgenes were identical to those reported earlier based on GUS reporter data (Somers and Quail, 1995; Goosey et al., 1997). The difference between the GUS and LUC reporter data concerning the activity of the PHYE promoter in the root tips can be explained by the different lengths of promoter fragments used in the constructs and/or by the different growth conditions. Moreover, similar to Clack et al. (1994), we were able to detect significant amount of PHYE transcripts in the roots of 1-week-old plants (data not shown), demonstrating that under our conditions, PHYE is expressed in the root tissue. On the one hand, these observations verify that luminescent data collected from our plants correctly reflect the regulation of these promoters. On the other hand, the data presented here offer a better resolution than those obtained by measuring mRNA accumulation of PHYC (Clack et al., 1994) and CRY1 and CRY2 (Ahmad and Cashmore, 1993; Hoffman et al., 1996). In addition, it is demonstrated clearly that CRY1 is expressed mostly in the aerial tissues (similar to CAB2), whereas the highest activity of CRY2::LUC is found in the leaf primordia and the root tip, and it is also clearly detected in other tissues including the cotyledons (similar to PHYA). The expression pattern of PHYC::LUC closely resembles that of CRY2::LUC+, with the difference that the highest level of expression was found in the cotyledons rather than in the shoot meristem.

Light-Regulated Expression

Taking advantage of the luciferase reporter system, we also determined the effect of light on the activities of the PHY and CRY promoters in vivo. To this end, plants were grown on LD cycles for 1 week, and then transferred to light or dark for the extended period of the measurement, during which luminescence data were collected in 1-h intervals. We found that the activities of the PHYA, PHYB, and CRY2 promoters are down-regulated, whereas the activities of the PHYC and CRY1 promoters are up-
regulated by light (Fig. 5). The same figure also shows that the expression of the PHYD and PHYE promoters was not affected significantly by the changes of light conditions. These observations are consistent with the results of earlier studies employing promoter:GUS reporters to study the light-regulated expression of the PHY promoters (Somers and Quail, 1995; Goosey et al., 1997). However, our data concerning the effect of light on the activities of the PHYC and CRY1, CRY2 promoters differ from previous results published by Clack et al. (1994), Ahmad and Cashmore (1993), and Lin et al. (1998). These authors reported that the accumulation of mRNA transcribed from these genes is unaffected by light. This apparent difference may be due to the different experimental setups (developmental stage of seedlings, frequency of sampling, and higher sensitivity of the assay used for the present study) or to the fact that light differentially regulates mRNA accumulation and promoter activity of these genes.

Diurnal- and Circadian-Regulated Expression

We demonstrate that the promoter activities of the PHY and CRY genes follow a diurnal rhythm and exhibit maximum expression in the light phase (Fig. 2). Furthermore, we show that these oscillations persist under LL and DD conditions with a period close to 24 h, proving that a circadian clock regulates the expression of these promoters (Figs. 3 and 4). Measurements of mRNA transcribed from these genes in seedlings transferred to LL indicate that the rhythmic expression is maintained at the level of mRNA accumulation (Fig. 6). These findings are consistent with our earlier results regarding PHYB (Kozma-Bognár et al., 1999) and with more recent data on CRY1, CRY2, and PHYA, PHYB mRNA levels derived from microarray experiments (Harmer et al., 2000; Schaffer et al., 2001).

We present the first evidence for the circadian regulation of PHYC, PHYD, and PHYE genes in this study. Based on our results, these genes can be used as new molecular markers to study circadian-regulated gene expression in Arabidopsis. However, the PHY and CRY genes form a special subgroup of the circadian markers because they can be placed in the input and the output pathways, as well. The temporal regulation of PHYC expression is different from that of the other PHY genes. The diurnal rhythm of PHYC::LUC activity indicates direct regulation by light rather than anticipation of light transitions. The circadian expression pattern of this construct is characterized by a very low amplitude with a peak at ZT 24 (versus ZT 10–12 for the diurnal rhythm) and by continuously increasing or decreasing expression levels in LL or DD, respectively. In contrast, PHYC mRNA accumulation exhibits a circadian rhythm with remarkable amplitude and peaks of expression at ZT 24, ZT 48, etc. These data suggest that the relatively strong light dependence of PHYC expression probably masks the effect of circadian regulation on the level of promoter activity and that circadian regulation affects accumulation of PHYC mRNA more dominantly, acting most probably on RNA stability.

Our data revealed striking similarities between the expression patterns of the PHYA and CRY2 promoters. The activities of these promoters follow the same spatial pattern. They are down-regulated by light and exhibit circadian oscillations with nearly identical characteristics, including amplitude and phase. Moreover, to enumerate further similarities, both proteins were shown to be light labile. These observations indicate that these photoreceptors are functioning primarily at low-light intensities.

Comparison of timing of maximum level expression of the various promoter:luciferase constructs allowed us to position the circadian phases of phytochrome and cryptochrome gene expression around a clock (Fig. 7). It is interesting that this comparison shows that the photoreceptor genes coding for relatively light-stable proteins (PHYC, PHYD, and PHYE) are intensively transcribed at the beginning or in the first one-half (PHYB and CRY1) of the light phase. It follows that the dramatic increase in light intensity at the beginning of the light phase can be accompanied by an increased accumulation of these receptors. We speculate that the newly synthesized masses of photoreceptors then mediate efficient adaptation of a variety of light-dependent processes (inhibition of hypocotyl and stem elongation, induction of genes coding for components of the photosynthetic machinery, and resetting the circadian clock) to these light conditions. By contrast, the expression of the PHYA and CRY2 genes coding for photolabile receptors reaches maximum close to the end of the light interval. It is characteristic of light signals, at
the oscillator even under relative constant conditions (McWatters et al., 2000). Our data suggest a similar (but not the same) role for the PHY molecules, but the underlying molecular mechanism remains to be elucidated.

MATERIALS AND METHODS

Promoter::Luciferase Fusions, Plant Materials, and Growth Conditions

The PHYA–E and CRY1 promoter fragments were obtained by PCR reactions performed on genomic DNA isolated from Arabidopsis (WT ecotype) plants. Unique restriction sites were designed at the 5’ and 3’ ends of the promoter fragments to facilitate cloning into the pUCV812 binary vector (Koncz and Schell, 1986) containing the modified luciferase (LUC+) reporter gene (Promega, Madison, WI) with the 3’-terminator sequences of the nopalinsynthase gene. All of the amplified fragments contained the entire 5’-untranslated region, but not the ATG of the corresponding genes. The fragment lengths and the unique restriction sites at the 5’ and 3’ ends were the following: PHYA, 2,357 bp, EcoRI-BamHI; PHYB, 2,292 bp, HindIII-BamHI; PHYC, 2,385 bp, EcoRI-Smal; PHYD, 2,310 bp, Sall-Smal; PHYE, 2,883 bp, HindIII-BamHI; and CRY2, 2,901 bp, HindIII-BamHI. The CRY1 promoter fragment, 1,004 bp in length bordered by EcoRI and Stul sites, was a gift of Anthony R. Cashmore (Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia). The identity of the promoter fragments was verified by restriction digestions and sequencing. The constructs were transformed into Arabidopsis (WT) plants by the Agrobacterium tumefaciens-mediated transformation method (Clough and Bent, 1998). Transformants were selected on Murashige and Skoog medium supplemented with 15 μg mL−1 of hygromycin. Ten to 15 independent transformants from each group were planted in soil, selfed, and the individuals of the F2 progeny were used for luminescence assays. It is notable that differences in spatial and temporal expression pattern among the independent transformant lines for a given construct have not been observed, but there were strong variations in the level of expression among the lines. Lines for the analysis were chosen to represent the entire range of expression levels: usually one low-, two medium-, and one high-expressing line of a given construct were included in each experiment.

Transgenic seeds carrying the PHYA–E::LUC+ and CRY1–2::LUC+ chimeric transgenes were surface sterilized, sowed on sterile Murashige and Skoog medium containing 3% (w/v) Suc, solidified with 1% (w/v) agar, and were then stratified at 4°C for 2 d. Seedlings were then grown in a phytocam (MLR-350, Sanyo, Gallenkamp, UK) at 22°C with 12-h-light (60-70 μM m−2 s−1, white fluorescent)/12-h-dark photoperiods for 7 d.

Luminescence Assays

Luciferase luminescence was measured by low-light video imaging using a liquid nitrogen-cooled CCD camera.
(Princeton Instruments, Trenton, NJ). Groups of 20 to 25 seedlings were germinated on Murashige and Skoog plates and were entrained as described above. Plants were sprayed with 5 mM luciferin solution (Biosynth AG, Switzerland) three times, 36, 24, and 12 h prior to the start of the imaging. Imaging started on the 8th d after germination at the beginning of the light phase (ZT 0). During the LL experiments, the seedlings were transferred to white LL (60–70 μM m^{-2} s^{-1}) at ZT 0. Alternatively, during the DD measurements, seedlings were transferred to DD at ZT 12. All experiments were performed at constant (22°C) temperature. Images were taken every 2 h (hourly for DD data acquisition), and exposure times were 15 min for PHYA,C::LUC, and exposure times were 25 min for PHYB,D,E::LUC. Brightness of areas containing groups of seedlings was measured by the MetaView software, corrected for background counts, and was normalized to the average value of luminescence of individual lines, as detected during the measurements. Normalized data were graphed as a function of time using Excel (Microsoft, Redmond, WA). Measurements were repeated at least three times on three or four independent transgenic lines for each construct with very similar results. Alternatively, to study the long-term light regulation of the various constructs, the luciferase activity of individual seedlings was measured in a Top-count NXT luminometer (Packard Instruments) as described by Carré and Kay (1995).

RNA Assays

Total RNA was extracted as described (Adam et al., 1994) from whole seedlings entrained for 7 d and was then transferred to LL as described above. Samples were harvested every 4 h. The abundance of the specific mRNAs was determined by RNase protection assays. The genespecific probes were obtained as short fragments of the coding region individual genes, amplified by PCR, and then cloned in pBluescript plasmid. The 5’- and 3’-end positions of the probe fragments referring to the nucleotides in the appropriate GenBank data files were: PHYA, 3,108 through 3,347 (X17341); PHYB, 3,348 through 3,565 (X17342); PHYC, 291 through 565 (X17343); PHYD, 2,888 through 3,177 (X76609); PHYE, 804 through 1,126 (X76610); CRY1, 111,223 through 111,397 (AL161513); CRY2, 1,261 through 1,542 (U43397); and UBQ10, 1,008 through 1,151 (L05361). Labeling of antisense RNA probes and subsequent steps of the RNase protection assays were performed as described by Adam et al. (1996). Thirty micrograms of total RNA was hybridized with the mix of the necessary gene probe and the UBQ10 probe. For CAB2 mRNA measurements, 15 μg of total RNA per lane was analyzed by northern hybridization using the CAB2 coding region probe (Millar et al., 1992). After exposure, blots were washed and rehybridized with the UBQ10 coding region probe. Radioactive signals were visualized in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and were quantified using the ImageQuant 1.1 software. Ratios between the individual PHY or CRY signals and the corresponding UBQ10 signals were calculated and normalized to the highest value. Experiments were performed two or three times and were highly reproducible; one representative set of data is shown in Figure 6.

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