Origins of Phytochrome-Modulated Lhcb mRNA Expression in Seed Plants

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The levels of Lhcb mRNA in higher plants are regulated by phytochrome, cryptochrome, and an endogenous circadian oscillator. To determine whether similar regulatory mechanisms operate in the ancient gymnosperm Ginkgo biloba, we measured Lhcb mRNA levels in seedlings in response to different light conditions. Removal of a diurnally oscillating light stimulus caused dampening of maximal Lhcb mRNA accumulation levels, with little change in periodicity. Although low fluence pulses of both red and blue light given to etiolated seedlings caused maximal accumulation of Lhcb mRNAs characteristic of the phasic/circadian response seen in flowering plants, the additional initial acute response seen in flowering plants was absent. The induction of Lhcb gene expression in both cases was at least partially reversible by far-red light, and appeared biphasic over a range of red fluences. Together, these data indicate that Lhcb genes in G. biloba appear to be regulated in a manner similar to that of flowering plants, whereas signaling and attenuation of mRNA levels through the photoreceptor systems and circadian clock show features distinct from those characterized to date. The implications for these findings are discussed in light of the evolution of circadian clock input signaling.

Plants perceive light via an array of photoreceptors whose absorption maxima together cover much of the visible light spectrum. Phytochromes are a family of photoreceptors that maximally absorb long wavelength red (R) and far-red (FR) light in the Pr and Pfr forms, respectively. Phytochrome proteins (phy), encoded by the phytochrome gene family PHYA-E in Arabidopsis, are synthesized in the inactive Pr form and converted to the active Pfr form by absorption of R (for review, see Quail, 1998). This activation can be reversed by immediate irradiation with FR, resulting in re-conversion of Pfr to Pr. Phytochromes absorb maximally in the R and FR regions, whereas absorption spectra for both forms indicate that they also efficiently absorb energy in the blue (B) light region of the visible spectrum (Mancinelli, 1994). For this reason, it has been difficult to determine whether responses elicited by B result from action through phytochrome or B/UV photoreceptors, such as the recently identified phototropin and cryptochrome receptors (for review, see Briggs and Huala, 1999). However, it has been possible to use photoreceptor mutants to determine which responses are under the control of particular photoreceptors (Chory, 1993; Koornneef and Kendrick, 1994). Responses regulated by both phytochrome and cryptochrome include the induction of transcription of nuclear genes encoding chloroplast polypeptides (for review, see Marrs and Kaufman, 1991; Thompson and White, 1991).

One family of genes whose expression is regulated by photoreceptors at the transcriptional level is that encoding the major light-harvesting chlorophyll a/b-binding proteins of photosystem II (LHC IIb, previously CAB2, polypeptides; for review, see Thompson and White, 1991). These genes, termed Lhcb genes, encode a small family of related polypeptides sharing a conserved structure of three membrane-spanning helices connected by more variable loop sequences and having variable N-terminal and C-terminal sequences (for review, see Green et al., 1991). The light-harvesting complex of photosystem II (which contains the LHC II polypeptides) is attached to the reaction center via a linker of the less abundant minor polypeptides. There are three major LHC IIb polypeptides, termed types 1, 2, and 3, encoded by Lhcb1, Lhcb2, and Lhcb3 genes, respectively. In addition to differing in sequence, the type 3 polypeptide has also been proposed to be closer to the reaction center than types 1 and 2 (Peter and Thornber, 1991). All three polypeptides function to bind chlorophylls a and b and carotenoids to conserve His residues in the helical region, and serve to anchor these pigments within the thylakoid membrane. Light energy absorbed the LHC II complex is ultimately funneled to the reaction center where it drives the photosynthetic light reactions.

In flowering plants, Lhcb genes are unusual among nuclear genes encoding chloroplast polypeptides in that they respond to R given in the very low fluence (VLF) range as well as the low fluence (LF) range (Kaufman et al., 1984; Nagy et al., 1986; Horowitz et al., 1988). Lhcb gene expression has also been shown
to respond to B (Gao and Kaufman, 1994), through a promoter region independent of R regulation (Folta and Kaufman, 1999). Individual Lhcb gene family members have been found to differ in the relative proportion of the response to R induced by VLF versus LF light (White et al., 1995). Genetic analysis in Arabidopsis indicates that the responses to VLF R and LF R are primarily controlled by the phyA and phyB photoreceptors, respectively (for review, see Quail et al., 1995). Because the roles of these photoreceptors were among the first to be elucidated using mutational analysis, their role in providing input for the circadian clock is well established (Somers et al., 1998; Strayer and Kay, 1999).

It is known that LF R illumination of etiolated Arabidopsis seedlings results in a transient accumulation of Lhcb mRNA occurring within 2 to 4 h of the light pulse. This rapid accumulation, termed the acute response, is followed by the decline and subsequent cycling of message levels with a periodicity of close to 24 h (Anderson and Kay, 1997). The first peak of this oscillation in Lhcb message levels occurs approximately 18 h after the light pulse and is thought to be a result of negative regulation by a circadian oscillator (Anderson and Kay, 1997). Modulation results in dampening of the amplitude of peak message levels, whereas the intrinsic clock mechanisms maintain a constant periodicity. Thus, a return to low steady-state message levels, approaching those of etiolated seedlings, is accomplished after several cycles. It has been demonstrated that the acute and circadian responses, as measured by increases in Lhcb mRNA levels in Arabidopsis, are genetically separable (Anderson et al., 1997). Moreover, the cycling of Lhcb mRNA levels in wheat (Triticum aestivum) is initiated by a VLF response (Nagy et al., 1993). A model for the regulation of Lhcb transcription has been proposed that involves phototransduction through the phyA and phyB photoreceptors regulating the acute response, followed by circadian clock attenuation of Lhcb mRNA levels acting downstream of phyB-E (Anderson et al., 1997).

Although photoreceptor and circadian regulation of gene expression have been extensively studied in flowering plant systems, particularly in the Arabidopsis model system, analyses of this kind have not been carried out for evolutionarily distant plant species. Data that suggest diurnal oscillations in light-harvesting complex mRNA levels in nonflowering plant species have been presented (Oberschmidt et al., 1995), but free-running diurnal oscillations indicating regulation by a circadian clock have not. Because we are interested in characterizing both phytochrome and cryptochrome function in gymnosperms, we decided to investigate photoreceptor and circadian regulation of gene expression in this group of organisms. Given that the developmental switch from skotomorphogenic (growth in the dark [D]) to photomorphogenic (growth in the light) programs is used to define photoreceptor function in flowering plants, we chose to study photoperception in the early diverging seed plant Ginkgo biloba.

G. biloba is the sole living survivor of the Ginkgo phyta division of the gymnosperms which emerged over 250 million years ago. Unlike the conifers, which can synthesize chlorophyll and initiate chloroplast development in complete darkness (Bogorad, 1950; Bogdanovic, 1973; Mukai et al., 1992), G. biloba etiolates in a manner similar to angiosperms. However, it accumulates very low protochlorophyllide amounts in D (Mariani and Rascio, 1982) and takes up to 2 weeks to green completely upon exposure to continuous white light (W). Type-specific probes have been prepared for Lhcb1, Lhcb2, and Lhcb3 genes and using these, it has been shown that the levels of all three mRNAs increase during greening (Chinn et al., 1995). Using the induction of Lhcb gene expression as an assay for phytochrome function, we set out to determine whether this nonflowering, seed-bearing plant has the responses characteristic of phytochrome action in flowering plants. In the present study, the regulation of Lhcb gene expression and the modulation of Lhcb mRNA levels in G. biloba have been investigated in more detail. We show that induction of Lhcb mRNA expression is under phytochrome regulation and that peak levels of Lhcb mRNA show diurnal oscillations in the absence of external light stimuli. Although phytochrome and circadian regulation of Lhcb mRNA levels in this system are similar to those characterized in Arabidopsis, G. biloba lacks the acute response and shows only a weak response to light given at the high end of the VLF range. These data provide information regarding the evolution of phytochrome-regulated responses in seed plants, and are interpreted in relation to well-characterized angiosperm light responses.

**RESULTS**

**Lhcb Gene Expression in G. biloba Is Modulated by the Circadian Clock**

The relative abundance of Lhcb1, Lhcb2, and Lhcb3 mRNAs in light-grown leaf was determined in a previous study (Chinn et al., 1995). Lhcb1 mRNA was found to be the most abundant species, representing approximately 0.14 femto mol μg⁻¹ total mRNA, whereas Lhcb2 and Lhcb3 mRNAs were found to be expressed at 10- to 15-fold lower levels (approximately 10 atto mol μg⁻¹ total mRNA). In the present study, we were interested in determining whether these genes respond differentially to light and/or the circadian oscillator, as well as the magnitude of their responses. After plants were entrained to a 12-/12-h W/D light cycle, diurnal oscillations in Lhcb mRNA levels could be detected. As seen in Figure 1, oscillations reached peak amplitude around the onset of the light period. Message levels subsequently declined during the light period, resulting in the lowest Lhcb1
mRNA levels early in the D period. After transfer to continuous D (at 24 h), the rhythmicity of these oscillations persisted with a period of approximately 24 h. After transfer to free-running conditions, peak Lhcb1 mRNA decreased in amplitude, a characteristic feature of Lhcb expression in higher plants. Although the period of these oscillations was not strictly maintained, persistent cycling of Lhcb1 transcript levels was apparent. Levels of both Lhcb2 and Lhcb3 mRNA also cycled with a pattern similar to that for Lhcb1 mRNA in entrained conditions; however, their relative levels (approximately 10-fold lower) only permitted demonstration of clear diurnal oscillation in free-running conditions (after transfer to complete D) for 1 to 2 cycles (data not shown).

**Lhcb Gene Expression in G. biloba Does Not Include an Acute Response**

When etiolated Arabidopsis seedlings are given a pulse of R, an initial pulse of Lhcb mRNA accumulation occurs within 2 to 4 h of the light treatment. This “acute response” has been demonstrated to result from phytochrome action and can be triggered by a single pulse of LF R light (Anderson and Kay, 1997). To determine whether this response occurs in G. biloba, we gave etiolated seedlings an LF pulse of R and measured subsequent Lhcb mRNA levels in the tips of these seedlings. As seen in Figure 2, levels of Lhcb1 mRNA remained virtually unchanged for the first 4 h after the LF R pulse. Accumulation of Lhcb1 mRNA increased substantially between 4 and 8 h post-light treatment in this experiment. Although accumulation began slightly earlier, between 2 and 4 h post-light treatment in one experiment, it is unlikely to have occurred as a result of an acute response because the onset of such accumulation begins within 1 h of the light pulse (Anderson et al., 1997). The timing of Lhcb2 and Lhcb3 mRNA accumulation was similar; however, the response magnitude appeared lower for these genes (data not shown). As was the case for Lhcb1 mRNA, the rise in message levels during this period appeared to be due to the onset of message accumulation that peaks later in the time course (see below).

**Lhcb Gene Expression in G. biloba Is Induced by a Pulse of Both LF R and LF B**

Although etiolated plants given an LF pulse of R did not accumulate significant levels of Lhcb transcript within 2 to 4 h, levels rose substantially after this period. Thus, we were interested in determining when the peak of this expression occurs. In subsequent experiments, expression therefore was monitored over a 48-h period, where tips were harvested at 4-h intervals for the first 24 h, and subsequently at 30, 36, and 48 h. As can be seen in Figure 3A, Lhcb1...
mA, Values (amol: 10$^{-18}$ mol) and returned to D. Aliquots of total RNA (5 μg) isolated from seedling tips and harvested at the indicated times, were blotted onto Hybond-N".

Figure 2. Acute levels of Lhcb1 transcript in response to a pulse of R. D-grown seedlings were given an LF pulse of R (10$^3$ μmol m$^{-2}$) and returned to D. Aliquots of total RNA (5 μg) isolated from seedling tips and harvested at the indicated times, were blotted onto Hybond-N and probed with α$^{32}$P-labeled antisense transcript prepared from an Lhcb1 gene-specific clone, as described in “Materials and Methods.” A, Values (amol: 10$^{-18}$ mol) were determined by generating calibration curves from blots of between 1 and 50 pg sense transcript from the Lhcb1 clone, probed in parallel with the experimental blots. Data were quantitated using a phosphorimager, are expressed on a per microgram basis, and represent the results from three independent experiments. B, Phosphorimager signals and photographs of ethidium bromide-stained RNA from the gel of one representative experiment (white squares) taken prior to blotting appears under the graph for signal intensity and loading comparisons.

mRNA levels in tips of seedlings treated with a single pulse of LF R increased over D levels to peak between 12 and 24 h. Levels then declined, returning to those seen for the tips of D-grown seedlings. This pattern of accumulation was also observed for Lhcb2 and Lhcb3 mRNAs (Fig. 3, B and C), although levels peaked approximately 10-fold lower than those for Lhcb1, consistent with their expression patterns in light leaf. This experiment was repeated three additional times, in which variations in the kinetics of mRNA accumulation were observed. For this reason, results from individual experiments are shown in Figure 3. However, in each experiment the maximal accumulation of all three Lhcb mRNAs occurred between 12 and 24 h after a pulse of R and was in the range of 50 to 200 amol μg$^{-1}$ total RNA for Lhcb1 and 5 to 20 amol μg$^{-1}$ total RNA for Lhcb2 and Lhcb3, illustrated by data from three independent light treatments.

The possible involvement of a B receptor was investigated by measuring accumulation of individual mRNAs in response to a pulse of LF B. Figure 3, D, E, and F, shows that mRNA accumulation for all three Lhcb genes was similar to that seen in response to a pulse of R, both in timing and the magnitude. Again, mRNA levels were quite variable between three independent experiments. Lhcb1 mRNA accumulated to between 50 and 200 amol μg$^{-1}$ total RNA, whereas Lhcb2 and Lhcb3 message levels accumulated to between 5 and 20 amol μg$^{-1}$ total RNA. In all three experiments, the maximum level of accumulation was between 12 and 24 h, similar to that for the response to an LF pulse of R. The most notable difference in the pattern of mRNA accumulation in response to LF pulses of R and B was between 30 and 48 h, where levels elicited in response to LF B did not return to D levels in all experiments (Fig. 3, D–F). This pattern was most visible for Lhcb1 mRNA measurements because levels of this message are far above the limit of sensitivity for this assay (approximately 1 amol μg$^{-1}$ total RNA). Because maximal accumulation of Lhcb mRNA was between 12 to 24 h after the light pulse in all experiments, a period of 18 h after light treatment (the median of this variation) was used for further experiments.

The Response to Both LF R and LF B Is at Least Partially Mediated by Phytochrome

To determine whether phytochrome is involved in the regulation of expression of Lhcb mRNAs in G. biloba, the ability of FR to reverse the effect of R was assayed. Consistent with the previous data, Figure 4A shows that Lhcb1 mRNA levels increased substantially over that seen in untreated D seedling tips in response to a pulse of LF R. Following an LF R pulse with LF FR caused at least partial reversal of this R-induced response. In addition, a small but statistically significant accumulation of Lhcb1 mRNA did occur after LF FR alone. Lhcb2 mRNA showed similar responses to R, FR, and R followed immediately by FR, as that seen for Lhcb1, although again the magnitude of the response to a pulse of R was lower (Fig. 4B), consistent with the previous experiments. It is surprising that Lhcb3 mRNA did not accumulate significantly in response to any of the light treatments (Fig. 4C). It is possible that the maximal level of Lhcb3 mRNA accumulation in these experiments did not peak at exactly 18 h, and because message levels of this gene do not respond strongly to a pulse of LF R, reversibility would not be measurable. However, the limit of detection of this mRNA and variation in accumulation kinetics precludes precise identification of this portion of the response.

It has been demonstrated that the phytochrome absorption spectrum extends into the B range (Mancinelli, 1994). Therefore, to determine whether the induction of these genes in response to B is due to phytochrome or is the result of the action of one or more B receptors, the ability to reverse the LF B
response by subsequent FR exposure was tested. Although an LF pulse of B was sufficient to induce accumulation of \textit{Lhcb1} and \textit{Lhcb2} transcripts, this response was at least partially reversed by FR (Fig. 4, D and E). Similar to the results obtained for FR reversal of \textit{Lhcb3} induction by R, levels of this mRNA did not respond significantly to any of the light treatments (Fig. 4F).

\textbf{Lhcb Gene Expression in \textit{G. biloba} Is Induced by a Pulse of LF Green Light}

To determine whether \textit{G. biloba} is able to respond to green light (and thus, whether it was possible that the green safelight could have been a source of the variability in the previous experiments), we measured \textit{Lhcb} levels in etiolated \textit{G. biloba} seedling tips in...
response to green light. Although significant accumulation of Lhcb1 mRNA (1.6–70.2-fold) over D levels was measured at 10^5 μmol m^-2 s^-1 green light, no difference over D levels could be detected for Lhcb2 and Lhcb3 mRNAs (data not shown). In addition, no difference could be detected for any of the three genes at fluence rates lower than 10^3 μmol m^-2 s^-1. Because the amount of green light received by seedlings during the course of LF R or LF B experiments was less than 1,000 times the levels necessary to elicit a measurable effect (and then only for Lhcb1), it was concluded that, whereas G. biloba can respond to green light wavelengths, the use of this green light source did not contribute significantly to experimental variation.

Lhcb Gene Expression in Response to R Is biphasic

Although all of the results from previous experiments were obtained using a safelight, VLF measurements require absolute darkness. Because G. biloba seedlings can respond to green light, albeit weakly, a safelight was not used for VLF measurements. The fluence response curves for each type of Lhcb mRNA in G. biloba are shown in Figure 5. All three mRNAs were detected at a comparable level of expression in D (1–10 amol μg^-1 total RNA, data not shown). Although levels of all three mRNAs responded to pulses of LF R at or above 10^5 μmol m^-2, response was significantly weaker to pulses of R below this threshold (at the high end of the LF range). For Lhcb1 and Lhcb2, the response is clearly biphasic and shows an initial increase in the range of 10^-2 to 10^-1 μmol m^-2. This slight increase in expression level, albeit weak, is sustained up to 10^3 μmol m^-2 for both Lhcb1 and Lhcb2. Although this type of biphasic accumulation pattern could not be observed for Lhcb3, expression does appear to be effected by R pulses as low as 10^3 μmol m^-2, the threshold for the VLF. Again, absolute accumulation levels were much lower for Lhcb2 and Lhcb3 messages (10–15 times lower than Lhcb1 levels, similar to previous experiments). Thus, the difference in absolute terms is small and given the inherent variability of the system, it is unclear whether Lhcb3 genes have altered fluence requirements from Lhcb1 and Lhcb2 genes in G. biloba.

DISCUSSION

In this study, we have: (a) demonstrated that Lhcb genes in G. biloba respond to LF, and weakly to VLF, R pulses; (b) shown that this response does not include acute Lhcb mRNA accumulation; (c) implicated the photoreceptor phytochrome in this response; (d) suggested that a B-light receptor may also play a role in Lhcb gene expression in G. biloba; and (e) shown that mRNA levels are regulated by a circadian oscillator.

We have utilized type-specific Lhcb probes to determine the relative and absolute expression levels of transcripts in G. biloba under various light conditions and in different developmental stages. The ultimate goal of this and parallel studies is to determine overlaps in the functional capacity of the photoreceptor systems in G. biloba and higher plants to help to define the origin of flowering plant photoreceptor function. Because Lhcb genes are among the most light-responsive genes in model systems, in terms of rapid response time and sensitivity to light (Thompson and White, 1991), they provide the most reliable indicator of the transcriptional capabilities of a plant system. In all organisms studied to date that have the capacity to respond to light in the VLF range, the induction of Lhcb gene expression is included in response capabilities.

All angiosperm Lhcb genes studied to date can respond to both a single pulse of LF R or LF B (for review, see Thompson and White, 1991). In a similar manner, G. biloba Lhcb genes respond to such light pulses within 24 h of the pulse. G. biloba Lhcb1, 2, and 3 genes differ, however, in the magnitude of their response, with Lhcb1 accumulating to approximately 10 times the transcript levels of Lhcb2 and Lhcb3. Although the levels of all three mRNAs are approximately equal in the D (from 1–10 amol μg^-1 total RNA), after LF pulses of either R or B, their levels reach peaks of 50 to 200 amol μg^-1 total RNA, 5 to 20

Figure 5. Fluence response curves for Lhcb1 (A), Lhcb2 (B), and Lhcb3 (C). D-grown seedlings (D) were given a pulse of R of the indicated fluence and returned to D for 18 h. Aliquots of total RNA isolated from seedling tips were processed as described in Figure 2, and data is expressed as a percent of the maximum pixel value obtained for each probe. Data represent the average ± se from four independent experiments.
amol μg⁻¹ total RNA, and 5 to 20 amol μg⁻¹ total RNA, for Lhcb1, 2, and 3, respectively. The response of Lhcb1 and Lhcb2 to both R and B was at least partially reversible by FR implicating the photoreceptor phytochrome in the induction of these genes under both LF R and LF B. The presence of a phytochrome pool with absorbance in both R and FR may also be implied by the slightly elevated Lhcb mRNA levels in response to LF FR pulses (Fig. 4). The relative effectiveness of both LF R and LF B appeared to be different for all three genes because the induction over D levels was severalfold greater for Lhcb1 than for Lhcb2, and Lhcb3 was statistically unresponsive. These results indicate that only the type 1 Lhcb genes in G. biloba respond strongly to light, and that regulation of these genes by light is unlike that of angiosperms in which types 1, 2, and 3 all respond strongly to light (White et al., 1992; Sigrist and Staehelin, 1994).

In Figure 1 of this study, we demonstrate that absolute levels of Lhcb1 mRNA oscillate between approximately 0.1 and 0.85 fmol μg⁻¹ total RNA in light leaf. Similar absolute expression levels have been reported for tomato (Lycopersicon esculentum), in which total steady-state Lhcb mRNA levels in light leaf was approximately 0.75 fmol μg⁻¹ total RNA (Kellmann et al., 1993). These values are in accordance with those determined in a previous study for the absolute value of Lhcb mRNA in light-grown G. biloba leaf (approximately 0.14 fmol μg⁻¹ total RNA; Chinn et al., 1995). However, through analysis of Lhcb expression in this and a parallel study (S. Christensen, E. LaVerne, G. Boyd, and J. Silverthorne, unpublished data), it is apparent that large variations in response timing and magnitude exist (a) between independent light treatments on the same batch of seedlings, and (b) between seedlings from different batches of seed. Although we set out to obtain statistical data for all experiments, such results could only be obtained for experiments carried out with the same batch of seedlings. Due to limitations in handling capacity and the order in which these experiments were performed, circadian and LF pulse data (Figs. 1–3) could not be presented with statistical analysis. However, the variation in response kinetics presented and discussed in this paper underscores the plasticity of the gene expression system for this organism, and should be anticipated in parallel studies of other gymnosperms.

Although variations in the timing and magnitude of the response to light pulses complicated statistical analysis, a reproducible difference in response kinetics could be observed between LF pulses of R and LF pulses of B. After accumulation levels peaked in response to LF R pulses, levels consistently returned to those seen for D-grown seedlings. However, after accumulation levels peaked in response to LF B pulses, levels did not consistently return to those of D-grown seedlings, and would frequently remain slightly elevated through the time period assayed (Fig. 3, D–F). Results from studies of seedlings treated with continuous monochromatic light indicate that B is more effective than R in promoting Lhcb expression in G. biloba stems (S. Christensen, E. LaVerne, G. Boyd, and J. Silverthorne, unpublished data). Together, these findings suggest that a B photoreceptor, like cryptochrome, may play a role in the induction of Lhcb gene expression throughout tips and bases of G. biloba seedlings.

Extensive molecular genetic studies of circadian-regulated gene expression have begun to elucidate the complex regulatory mechanism underlying diurnal rhythmicity. This mechanism is thought to be defined by a central oscillator resulting from auto-regulatory inhibition involving positive factors that act on genes encoding negative factors that feed back to regulate their own expression (Strayer and Kay, 1999). Thus, transcription factors including CCA1, LHY, and TOC1 in Arabidopsis (Schaffer et al., 1998; Wang and Tobin, 1998; Strayer et al., 2000) play central roles in mediating circadian period and amplitude. Fundamental to environmental entrainment of the clock are inputs from various photoreceptors. The recent identification of ZTL, FKF1, and GI in Arabidopsis (Park et al., 1999; Nelson et al., 2000; Somers et al., 2000) suggests that some clock components may function as input photoreceptor, oscillator component, and/or coupling mechanisms to ubiquitin-dependent proteolysis. Although such specific clock components have yet to be identified in gymnosperms, the findings presented here allow us to speculate about the evolution of phytochrome and cryptochrome in providing input to the circadian clock, in seed plants.

In a parallel study (S. Christensen, E. LaVerne, G. Boyd, and J. Silverthorne, unpublished data), we describe the FR high irradiance response in G. biloba. The presence of this response suggests that this function of phyA (and perhaps also the sensitivity to high-end VLF light suggested in this study) had evolved prior to the divergence of G. biloba from the origin of seed plants. The finding that Lhcb gene expression in G. biloba has a weak VLF component, however, suggests a difference in the sensitivity between the G. biloba phytochrome system and that of the model plant Arabidopsis. In addition, the absence of an acute response in Lhcb gene expression, in combination with the R/FR reversibility of the LF response, implies that the G. biloba phytochrome system does not support rapid, transient gene expression. Thus, it is likely that the acute response evolved after the divergence of flowering plants, and developed as part of the rapid developmental strategy of angiosperms. Although we have identified both phytochrome and cryptochrome homologs in this organism (S. Christensen, J. Silverthorne, and M. Wada, unpublished data), the specific role of these photoreceptors in the entrainment of the circadian clock has...
not been investigated. However, our data indicate that the ability to sense and respond to different fluence rates of both continuous R and B is present in *G. biloba*. Thus, it would appear that the collective ability of the R-and B-sensing systems in providing input to the circadian clock in flowering plants had evolved prior to the divergence of *G. biloba* from the origin of seed plants. Because both phyA- and phyB-type functions are present in *G. biloba*, it may be that the situation is similar to Arabidopsis, where a phyA-like molecule provides input to the clock under continuous LF R conditions, whereas a phyB-like receptor provides such information under continuous high fluence R conditions (Somers et al., 1998). A single phytochrome species alternatively might provide input to the clock under a diverse range of fluence conditions.

Although it has been demonstrated that there is a functional dependence of the cryptochrome receptor on phytochrome in Arabidopsis (Ahmad and Cashmore, 1997), the interaction of the two systems appears to differ between flowering and nonflowering plants. In *Adiantum capillus-veneris* gametophytes (i.e. fern spore germination), B inhibits the effects of R on the first cell division, whereas R inhibits the second and subsequent cell divisions promoted by B (Furuya et al., 1997). Because seed germination in flowering plants is effected positively by both R and B, the regulation of these responses is clearly disparate. The extent of interaction between the two photoreceptor systems in *G. biloba* has not been determined; however, the minor difference between response to R and B observed in this analysis would indicate that both systems function to promote the expression of light-responsive genes, such as those encoding the LHC IIb polypeptides. The co-action of these two light-perceiving systems in early seedling development is addressed in a parallel study (S. Christensen, E. LaVerne, G. Boyd, and J. Silverthorne, unpublished data), whereas the molecular nature of phytochrome and cryptochrome species in *G. biloba* is currently under investigation.

**MATERIALS AND METHODS**

**Ginkgo biloba Growth Conditions and Light Treatments**

Seed was purchased in the Chinatown district of San Francisco. A small portion of the seed coat was removed and the seeds were allowed to imbibe water for 24 h in D, then germinated in vermiculite in D. The tissue was collected under a dim-green safelight (λ<sub>max</sub> 506 nm) in all experiments, with the exception of the fluence response curve and green light pulse experiments which were carried out in complete D. D controls for R pulse and B pulse experiments were harvested immediately prior to light treatment for that set of plants. D controls for FR reversal, fluence response curve, and green light pulse experiments were handled and harvested in the same manner as the tissue receiving light treatment. For these experiments, entire seedlings were removed from the vermiculite and placed horizontally under the light source for treatment. The seedlings were subsequently returned to D in a vertical position in a beaker with their roots submerged in water.

Light sources for W, R, and FR are described by Peer et al. (1996). B was supplied by filtering light from two B-emitting fluorescent tubes (F40B, General Electric, Hendersonville, NC) through two layers of plexiglas (blue no. 2424, Rohm and Haas, Chicago Heights, IL). The R and B pulse times were generated by giving seedlings an LF pulse of R or B (10<sup>3</sup> μmol m<sup>−2</sup>) and returning the seedlings to D until harvest. Fluence response, FR reversal, and green light pulse tissue was treated with the defined quantity of light and returned to D for 18 h. Irradiation times for R were: 10<sup>−4</sup> and 10<sup>−3</sup> μmol m<sup>−2</sup>, 1 s; 10<sup>−2</sup> to 1 μmol m<sup>−2</sup>, 10 s; 10 to 10<sup>3</sup> μmol m<sup>−2</sup>, 1 min; and 10<sup>4</sup> μmol m<sup>−2</sup>, 10 min. Irradiation times for the LF pulse of B and FR were 100 s and 10 min, respectively. Irradiation time for green pulses were: 1 to 10 μmol m<sup>−2</sup>, 50 s; and 10 to 10<sup>2</sup> μmol m<sup>−2</sup>, 500 s. Tips of the etiolated seedlings (as defined by the position of the first bract, 0.5–1.5 cm from the top) were collected rather than entire stems due to the differential greening pattern seen along the length of the stems (Chinn et al., 1995). A minimum of 10 seedlings was used for each light treatment.

The initiation of seed germination in *G. biloba* is variable within a given batch of seed. This results in a range of seedling sizes from 1 to 20 cm within 6 weeks of growth in D. We investigated the possibility that seedling size/age could influence the response to both LF R and LF B; however, no correlation could be made. Thus, variations in absolute Lhcb expression levels between experiments are likely due to inherent variability of *G. biloba* to either sense or respond to such light treatments. Thus, a range of Lhcb mRNA levels are reported in the text.

Circadian rhythm experiments were performed by growing D-germinated seedlings in a 12-/12-h W/D cycle for at least 4 weeks. Leaves were collected from a minimum of three seedlings per time point every 4 h, starting at the beginning of a light cycle. Plants were transferred to continuous D at the end of that light period, and subsequent tissue collection was done in complete D. Tissue harvested from all the experiments previously described was immediately frozen in liquid N<sub>2</sub> and stored at −80°C until use.

**RNA Isolation and Northern-Blot Analysis**

Total RNA was prepared as described previously (Chinn and Silverthorne, 1993) with the addition of a final step to remove remaining particulate matter of centrifugation at 15,000 rpm, 15 min, 4°C, followed by selective precipitation of mRNA with 2 M LiCl. The resulting pellet was then washed with 80% (v/v) ethanol and resuspended in 20 μL sterile distilled water. RNA concentrations were determined by triplicate A<sub>260</sub> measurements using a UV-160 spectrophotometer (Shimadzu, Canby, OR). RNA samples were analyzed by electrophoresis on 1% (w/v) agarose gels in MOPS [3-(N-morpholino) propane sulfonic acid] buffer (20 mM MOPS, 1 mM EDTA, and 5 mM sodium acetate, pH
7.0) containing 6.7% (v/v) formaldehyde (Lehrach et al., 1977). Gels that were to be probed for Lhcb1 and Lhcb2 mRNAs were electrophoresed for 1 h at 100 V, whereas those to be probed for Lhcb3 were electrophoresed for 2 h for complete resolution of these mRNAs from 18S rRNAs. Gels were rinsed in distilled water (three times for 20 min each), RNA visualized using ethidium bromide (1 µg mL⁻¹), photographed, and blotted directly onto Hybond-N nylon membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ), according to the manufacturer’s protocol. Hybridization to [α³²P]-labeled type-specific antisense 3'-untranslated region (3'-UTR) RNA probes were performed as described by Chinn et al. (1995). The membranes were washed for 1 h at the following temperatures to obtain probe specific hybridization; Lhcb1 3'-UTR, 70°C; Lhcb2 3'-UTR, 65°C; and Lhcb3 3'-UTR, 55°C. Excess nonspecific background hybridization was minimized by a final wash with 1 µg mL⁻¹ RNaseA. The absolute amounts of each type of mRNA were quantitated using a calibration curve with 1 µg mL⁻¹ RNaseA. The absolute amounts of each type of RNA were quantitated using a calibration curve with 1 µg mL⁻¹ RNaseA.

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