

Thesis of Ph.D. dissertation

**THE ROLE OF THE PHYTOCHROME B
PHOTORECEPTOR IN THE REGULATION OF THE PLANT
CIRCADIAN CLOCK AND CIRCADIAN RHYTHMS**

Andrea Palágyi

Supervisor: Dr. László Kozma-Bognár

School of Biology, University of Szeged

Biological Research Centre of H.A.S.

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BACKGROUND

Circadian clocks regulate many rhythmic cellular and physiological processes and allow a wide range of organisms to adapt to the predictable daily changes in the environment (e.g. day/night cycles). At the core of the clock, components of the central oscillator (clock genes/proteins) mutually regulate their expression/activity via multiple feedback loops that results in an autonomous, self-sustained ~24h oscillation. The core oscillation is relayed to diverse clock-controlled processes (gene expression, physiology, behavior) via the output pathway. As the period length of the core oscillation always deviates from 24h, the clock must be re-synchronized to the environmental cycles regularly in order to provide precise temporal information. In the nature, this resetting occurs on a daily basis in response to periodic environmental cues (e.g. changes in temperature and light conditions). Light is absorbed by specialized photoreceptors and signals are forwarded by the input pathway to modulate the pace and the phase of the oscillator. The plant (*Arabidopsis thaliana*) circadian oscillator is supposed to consist of three inter-locked feedback loops. In the first loop (central or coupling loop) the morning-expressed CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)/LATE ELONGATED HYPOCOTYL (LHY) transcription factors inhibit the expression of the *TIMING OF CAB EXPRESSION 1 (TOC1)* gene; conversely, the evening-expressed TOC1 positively regulates the transcription of *CCA1/LHY*. In the second loop (evening loop) the predicted factor Y induces *TOC1* expression during the afternoon/evening, while TOC1 represses Y during the night. It has been demonstrated that GIGANTEA (GI) functions as a component of Y. In the so-called morning loop CCA1/LHY up-regulate the *PSEUDO RESPONSE REGULATOR 7/9 (PRR7/9)* genes (homologs of *TOC1*) in the morning and PRR7/9 proteins down-regulate *CCA1/LHY* expression during the day. The coordinated function of the three loops is required to generate the ~ 24 h basic oscillations in *Arabidopsis*.

Light signals are perceived and transduced to the clock by specialized photoreceptors, including members of the blue light absorbing cryptochrome (CRY) and the red/far-red light sensing phytochrome (PHY) families. The *Arabidopsis* PHY photoreceptor family consists of five members (PHYA-E), which function as molecular light switches. In the dark, PHYs are present in their inactive red light absorbing (Pr) form ($\lambda_{\max} = 660$ nm). After capturing a photon by the covalently bound linear tetrapyrrol chromophore, they are converted to the active far-red light absorbing conformer (P_{fr}), which initiates downstream signaling events in the cytosol or in the nucleus. The active P_{fr} form is converted to Pr by far-red light ($\lambda_{\max} = 730$ nm). The P_{fr} conformers of PHYs are translocated to the nuclei, where they form characteristic nuclear bodies (NBs). The exact composition and function of NBs is not yet known, but they may represent multi-protein complexes, where PHYs interact with transcription factors and other regulatory proteins to control expression of light-induced genes.

Involvement of PHYA, B, D and E has been demonstrated in the function of red light input to the clock. PHY signaling affects transcription rate, mRNA and protein turnover of several clock components, although the signal transduction pathways linking these events with light-activated PHYs are largely unknown. On the other hand, several reports have demonstrated that PHY mediated continuous red light signals inversely affect the free-running period length in a fluence rate-dependent manner (parametric entrainment) and discrete red light pulses elicit characteristic phase shifts of the clock and overt circadian rhythms (non-parametric entrainment). The role of PHYB has been evidenced in both processes. It has been shown by several research groups that the absence of PHYB function (i.e. in *phyB* mutants) results in long period of the rhythmic transcription of the *CHLOROPHYLL A/B BINDING 2* (*CAB2*) gene at higher fluences of continuous red light, whereas no period alterations were observed in *phyB* mutants in continuous white light. Interestingly, *phyB-9* but not *phyB-1* displayed an early phase of *CAB2:LUC* expression under these conditions. This could indicate allele- and/or ecotype-specific effects of PHYB function or could reflect the

functional interaction of the PHY and CRY mediated signaling to the clock in white light, the precise spectral composition and fluence rate of which is difficult to reproduce. Using leaf-movement assays, it also has been shown that PHYB is required for red light induced phase shifts. It should be noted, that in all the experiments reported so far the function of PHYB as circadian input photoreceptor was evaluated on the basis of altered output rhythms (*CAB2*, *PHYB* expression, leaf-movements), but the rhythmic expression of core clock components has not been tested yet.

PHYB was first identified by the characteristic photomorphogenic phenotype of *phyB* mutants, which show elongated hypocotyls, the lack of hypocotyl hook opening and reduced cotyledon expansion in red light. The molecular pathway by which PHYB controls photomorphogenesis involves the nuclear translocation of PHYB P_{fr} and its interaction with regulatory proteins. The initiation of flowering is a complex developmental process in *Arabidopsis* partly controlled by the rhythmically expressed transcriptional activator CONSTANS (CO) protein. Light signals mediated by PHYB destabilize CO protein specifically in the morning. As a result, *phyB* mutants flower earlier in any light conditions tested so far.

The characteristic domain structure of PHYs and analysis of *phy* mutants displaying altered light sensing or signaling capabilities suggested that the N-terminal domain is required for light absorption, whereas downstream signaling cascades are activated by the C-terminal domain. However, it has been shown, that N-terminal fragments of PHYB containing 651 or 450 amino acids (aa) of the receptor fused to the bacterial β -glucuronidase (GUS) protein (providing dimerisation motifs) and nuclear localization signals (NLS) were biologically active in regulating photomorphogenesis. These reports have also demonstrated that the 651, but not the 450 aa N-terminal fragment retained the function of PHYB in controlling flowering. These results indicated that the histidine kinase-like subdomain, which is located within the C-terminal part of PHYB is dispensable for downstream signaling mediating photomorphogenesis and flowering.

However, information on the circadian function of N-terminal fragments of PHYB of different lengths or the identification of the minimal PHYB fragment required for certain functions of the receptor is still missing.

RESEARCH OBJECTIVES

Our research group is interested in the functional interaction of signaling pathways mediated by the circadian clock and the phytochrome receptors in *Arabidopsis thaliana*. The experiments presented in this study were designed to provide novel and detailed information on the process through which the red light photoreceptor phytochrome B (PHYB) regulates the circadian clock. The main objectives were::

1. To determine the effect of PHYB on the function of the clock by quantitative analysis of period length and phase of rhythmic expression of output genes and core clock genes in monochromatic red and white light.
2. To determine the particular domains of PHYB responsible the regulation of photomorphogenesis, flowering time and the circadian clock under different light conditions.

RESEARCH METHODS

- Culturing *Arabidopsis thaliana* plants under sterile and greenhouse conditions
- Molecular cloning techniques
- Plant genomic DNA extraction
- Plant total RNA extraction
- Quantitative Real-Time PCR assay
- Western-blotting
- Generation of transgenic plants

- Light, fluorescence and confocal microscopy
- *In vivo* luciferase enzyme activity measurements

RESULTS AND DISCUSSION

Phytochrome B (PHYB) is the dominant red/far-red light absorbing photoreceptor in light-grown *Arabidopsis* plants. PHYB provides signals to regulate photomorphogenesis, to control flowering and to entrain the circadian clock. Consequently, *phyB* mutants display long hypocotyls, accelerated flowering and long periods of circadian rhythms under specific light conditions. The PHYB receptor folds into two main domains: the chromophore-bearing photosensory N-terminal domain and the C-terminal domain containing a histidine kinase-like subdomain and protein motifs for dimerization and nuclear translocation. It has been demonstrated that dimers of the N-terminal domain of PHYB possess full PHYB function regarding photomorphogenesis and flowering time determination if they are targeted to the nucleus by added foreign NLS motifs. In other words, the histidine kinase domain is dispensable for these functions of PHYB and the main role of the C-terminal part is to provide a platform for dimerization and to control the entry of the protein in the nucleus.

In order to test the function of the N-terminal domain to provide light signaling to the circadian clock, we expressed the 651 aa N-terminal fragment fused to YFP, dimerization domains and NLS or NES protein motifs (B651-NLS and B651-NES, respectively) in the *phyB-9* background, which lacks any PHYB functions. The fusion proteins, including the YFP-tagged full length PHYB control (BFL) were expressed at comparable levels and showed the expected subcellular localization. The light input pathway to the circadian oscillator mediates two modes of entrainment depending on the duration of illumination. In constant light conditions, the activity of the input pathway shortens the free-running period length with increasing light intensity (parametric entrainment). In constant darkness, however, short light pulses elicit discrete phase advances or delays of the oscillator and overt

circadian rhythms (non-parametric entrainment). Red light signals absorbed by PHYB contribute to both types of entrainment. We found that rhythmic expression of core oscillator genes has longer period in *phyB-9* plants compared to wild type in continuous red light at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ fluence rate. On the other hand, in absence of PHYB function, red light pulses trigger smaller phase shifts of the oscillator. Our results demonstrated that B651-NLS was as effective to restore these phenotypes of the *phyB-9* mutant as the full length PHYB. Interestingly, BFL (and B651-NLS) showed significant increase in the amplitude of mRNA rhythm of *CCA1*, but did not cause shorter periods. It has been reported that over-expression of PHYB results in short period of *CAB:LUC* expression in continuous red light and in early flowering under both SD and LD conditions. The lack of circadian and flowering time phenotypes of BFL can be explained by similar PHYB levels in BFL and WT plants, in contrast to lines used in the cited studies, which show at least 15-fold over-expression of the PHYB protein. In contrast to BFL and B651-NLS, B651-NES was unable to restore PHYB function to regulate rhythmic *CCA1* or *TOC1* expression. These results demonstrate that the N-terminal domain of PHYB is capable to mediate all aspects of entrainment of the circadian clock and this function requires nuclear localization of PHYB. The data presented here also verify previous reports, since B651-NLS, but not B651-NES restored the photomorphogenic and flowering phenotypes of *phyB-9*.

The molecular mechanism by which nuclear localized PHYB mediates entrainment is unclear, but most likely involves interaction of PHYB with regulatory proteins, which indirectly or directly affect the oscillator. Among such clock-affecting proteins, ZEITLUPE (ZTL) and EARLY FLOWERING 3 (ELF3) have been shown to interact with PHYB in yeast or *in vitro*. ZTL is an F-box protein, which targets TOC1 proteins for ubiquitination by Skp1/Cullin/F-box (SCF) type E3 ubiquitin ligases and for subsequent degradation and severely affects the free-running period length. EARLY FLOWERING 3 (ELF3) is a clock-controlled nuclear protein, which attenuates light-induced resetting of the clock. However,

both ZTL and ELF3 bind to the C-terminal domain of PHYB, which is dispensable for circadian function in red light according to our data; therefore, these interactions have no relevance to entrainment under these conditions.

In fact, PIF3 has been shown to interact with the N-terminal domain of PHYB P_{fr} and also with TOC1. Since PIF3 potentially binds to the G-box element located in the promoter of *CCA1* and *LHY* genes, the PHYB-PIF3-TOC1 complex could provide a mechanism for directing regulatory light signals to certain core oscillator genes. It has been shown that PIF1, 4 and 5 also interact with TOC1. However, misexpression of PIF3 or PIF5 does not affect entrainment of the plant circadian clock. Although the lack of such phenotypes could be explained by possible redundant co-action of several PIF3-like transcription factors, PIFs are probably not the terminal components of PHYB-mediated red light input to the clock, but rather represent components of clock-controlled output processes (e.g. rhythmic hypocotyl elongation).

It has been reported that a short fragment containing 450 aa of the N-terminal domain of PHYB mediates photomorphogenic responses in the nucleus. We created *phyB-9* plants expressing this PHYB derivative in fusion with dimerization and NLS signals (B450-NLS). Analysis of these transgenic lines verified that the PHY subdomain of the N-terminal part of PHYB is not required for the inhibition of hypocotyl elongation and revealed that this subdomain is also dispensable for the regulation of period length. We demonstrated that B450-NLS rescued the wild type expression pattern of *CCA1* and *TOC1*. Unlike the photomorphogenic and circadian phenotypes, the early flowering phenotype of *phyB-9* was not restored by B450-NLS. B450-NLS plants grown in long day (16h light / 8h dark) flowered at the same time as *phyB-9* mutants. This result is consistent with those reported earlier. This observation indicates that the PHY subdomain may provide a binding site for a yet unidentified factor, which is necessary to transmit signals eventually destabilizing CO protein and delaying flowering. The facts that PHYB acts on CO protein levels and that the flowering phenotype of *phyB* mutants is independent of

photoperiods demonstrate that early flowering of *phyB* mutants does not arise from altered clock function. Our results further support this conclusion, because B450-NLS plants generate wild type circadian rhythms, yet display the early flowering phenotype of *phyB-9*.

In order to define the minimal N-terminal PHYB fragment, which is still functional in the nucleus, we created transgenic plants expressing a 410 aa derivative of PHYB (B410-NLS). Despite the fact that this version is only 40 aa shorter than B450-NLS, B410-NLS was unable to complement any phenotypes of *phyB-9* in any assays. It must be noted that the effect of both B651-NLS and B450-NLS was light dependent and phenotypes indicating constitutive light-independent signaling were not observed in these plants in darkness. This means that light-induced conformational change is a prerequisite of the biological function of these derivatives. In order to undergo light-induced $P_r \leftrightarrow P_{fr}$ conversions, the chromophore must be attached to the PHYB apoprotein. Zn-blots were used to test the chromophore binding capability of the different PHYB derivatives. We provided evidence that B651 and B450 were able to autoligate the chromophore. In contrast, B410 failed to incorporate the chromophore, indicating that the deletion probably affected the function of the bilin lyase domain.

Quantitative analysis of clock gene expression in the *phyB-9* mutant revealed an unexpected complexity in the function of the light input to the circadian oscillator. The plant oscillator consists of three interlocked transcriptional/translational feedback loops. The “morning” loop is operated by interactions among *CCA1/LHY* and *PRR7/9* genes, the “evening” loop is based on the cross-regulation between *TOC1* and *Y/GI* and the two loops are coupled by a central loop formed by *CCA1/LHY*, *TOC1* and a yet unidentified X factor. In wild type plants these loops are coupled together, which is illustrated by stable and constant phase relationships of expression of these genes under free-running conditions. Theoretically, the morning and evening loops could be uncoupled from each other in certain conditions, for example as a result of miss-function of X. Our data demonstrated

that loss of PHYB function affect the pace of the morning and evening loops differently. Expression of *CCA1* and *PRR9*, components of the morning loop, show significant long period rhythm in *phyB-9* over a wide range of fluence rates of continuous red light, but *TOC1* and *GI* expression display less significant period lengthening. Importantly, at lower fluences of red light, the pace of the evening loop is not affected by the lack of *phyB-9*, whereas the morning loop runs at a lower pace in the same conditions. These data demonstrate that in these conditions the coupling between the morning and evening loops has been weakened in *phyB-9*. Although the exact molecular mechanism of this phenomenon and the role of PHYB in the apparent decoupling remain unclear, our results shed light on the complexity of the function of the light input to the plant circadian oscillator. This complexity was further expanded by the analysis of circadian gene expression in the *phyB-9* mutant in continuous white light. Our data showed that PHYB has nearly opposite effects on the pace of the clock in red and white light, since circadian period lengths in the *phyB-9* mutant are generally lengthened or shortened in continuous red or white light conditions, respectively. On the other hand, it has been demonstrated that the *phyB-1* mutation has no effect on period length in monochromatic blue light. The cool white fluorescent tubes used in this experiment provide light enriched in blue light, but still emit red light capable of PHYB activation. This is indicated by the long hypocotyls of *phyB-9* seedlings under this condition. It follows that the short period phenotype *phyB-9* is apparent upon simultaneous activation of the red and blue light signaling pathways to the clock, which could be explained by a negative effect of PHYB on blue light signaling. The unexpected short period phenotype in continuous white light has been observed for leaf-movement rhythms in the quintuple phy mutant, also suggesting that blue light input to the clock is attenuated by the action of phytochrome(s). Since PHYB appears to have a positive effect on CRY2 signaling, other routes of blue light input may be affected. However, molecular details of this particular functional interaction between red and blue light signaling to the clock remain to be

elucidated. We showed that BFL, but not B651-NLS was able to complement the short period phenotype of *phyB-9* in white light. Since the BFL and B651-NLS proteins were expressed at similar levels, the lack of complementation by B651-NLS is most probably due to absence of the C-terminal domain of PHYB.

In conclusion, our data suggest that the nuclear localized N-terminal domain of PHYB is fully functional in regulating photomorphogenesis, flowering time and red light-dependent entrainment of the circadian clock. However, the C-terminal domain is essential for proper circadian entrainment in white light conditions. To explain the requirement of the C-terminal domain for this process, we propose that this domain mediates integration of the blue and red light signaling pathways to the clock.

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LIST OF PUBLICATIONS

Publication used in the thesis:

Palágyi, A., Terecskei, K., Ádám, É., Kevei, É., Kircher, S., Mérai, Zs., Schäfer, E., Nagy, F., Kozma-Bognár, L. (2010) Functional analysis of amino-terminal domains of the photoreceptor phytochrome B. *Plant Physiology* 153, 1834-1845

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