THE ROLE OF THE PHYTOCHROME B PHOTORECEPTOR IN THE REGULATION OF PHOTOPERIODIC FLOWERING

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LIST OF PUBLICATIONS

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Other publications:

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Kozma-Bognar L,**Hajdu A,** Nagy F.(2012) : Light-regulated gene expression in yeast. *Methods in Molecular Biology***813**:187-93.

Fehér B, Kozma-Bognár L, Kevei E, **Hajdu A,**Binkert M, Davis SJ, Schäfer E, Ulm R, Nagy F. (2011) :Functional 8-controlled interaction of the circadian clock and UV RESISTANCE LOCUS UV-B signaling pathways in *Arabidopsis thaliana***.** *Plant Journal*.**67**:37-48
IF: 6.16

Sorokina O*, **Kapus A***, Terecskei K, Dixon LE, Kozma-Bognar L, Nagy F, Millar AJ.(2009) : A switchable light-input, light-output system modelled and constructed in yeast**.** *Journal of Biological Engineering*.17;3:15
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BACKGROUND

Timing of reproduction is vital for any living creatures, but is particularly important for sessile organisms, like plants. Flowering is regulated by developmental signals, but also by environmental cues like day length, quality of light or abiotic stress. Many plants use day length as an indicator of the actual season of the year, to be preferred or avoided as the time to set seeds. *Arabidopsis thaliana*is a facultative long day plant meaning that flowering is initiated much earlier in long day (LD) conditions (e.g. 16 h light / 8 h dark cycles) than in short day (SD) conditions (e.g. 8 h light / 16 h dark cycles). Photoperiodic time measurement in Arabidopsis is based on the functional interaction of the endogenous circadian clock and environmental light signals mediated by special photoreceptors.

The circadian clock rhythmically regulates the transcription of *CONSTANS* (*CO*) in a way that high level of *CO* expression coincides with light only in the evenings of long days.At this time of the day, photoreceptors phytochrome A (phyA) and cryptochomes (CRY1 and CRY2) stabilize the CO protein by inhibiting the function of the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) E3 ubiquitin ligase. Consistent with the action spectra of these receptors, far-red and blue light are the most effective in this process. In contrast, phyB, the dominant red light-absorbing receptor in light-grown plants, promotes degradation of CO during the first half of the day most probably by enhancing the activity of a yet unidentified ubiquitin ligase.The net effect of these regulatory processes is the accumulation of CO proteins in the evening of long days. CO is a Zinc-finger B-box type transcription factor that induces the expression of *FLOWERING LOCUS T* (*FT*). The FT protein is considered as the long-searched florigen, which is produced in the leaves, but moves to the shoot apical meristem through the phloem to the shoot apical meristem, where it promotes the transition from the vegetative to reproductive state by the activation of floral integrator genes. The photoperiodic regulation of flowering can be affected by alterations in the function of the

circadian clock and/or the photoreceptors. For example, short period clock mutants show an early phase of circadian rhythms in light/dark cycles. Most of these mutants flower early in SD conditions, because the peak of rhythmic CO transcription is shifted from the night to the daytime allowing phyA/CRY1-2-dependent stabilization of CO and subsequent activation of *FT*. On the other hand, mutants lacking phyA or CRY1-2 function flower late in LD conditions, because of the absence of CO stabilization. In *phyB* mutants, however, flowering is accelerated, that is caused by accumulation of CO and high-level transcription of *FT* during the day.

RESEARCH OBJECTIVES

It has been known for two decades that overexpression of phyB results in early flowering especially in SD conditions that is in sharp contrast to the well-defined role of the receptor in the regulation of flowering. The primary aim of our work was to solve this paradox by revealing the molecular mechanism by which phyB overexpression accelerates flowering.

RESEARCH METHODS

- Culturing *Arabidopsis thaliana* plants under sterile and greenhouse conditions
- Molecular cloning techniques
- Plant genomic D NA extraction
- Plant total RNA extraction
- Quantitative Real-Time PCR assay
- Western-blotting
- Generation of transgenic plants
- *In vivo* luciferase enzyme activity measurements

These observations suggest that pace of the clock is much less sensitive to the amount of phyB Pfr than the control of hypocotyl elongation.

CONCLUSIONS

Our data collectively suggest that the net effect of phyB on CO turnover is determined by the combination of three factors: the time of the day, the level of phyB Pfr and the particular ubiquitin ligase controlled by phyB Pfr. In the first half of the day phyB appears to promote degradation of CO, independent of overexpression. phyB enhances the function of the unidentified ubiquitin ligase that overrides the effect of inhibition of the COP1-SPA complex. In the second half of the day and around dusk the function of the unidentified ubiquitin ligase is less dominant, but the elevated *FT* levels in the *phyB-9* mutant indicate that this function is not totally absent yet. Overexpression of phyB induces rather than reduces *FT* levels indicating the increasing effect of the inhibition of COP1-SPA. In the night, the COP1-SPA complex has the prevailing effect on CO stability, thus phyB overexpression results in massive *FT* induction, whereas in wild type plants levels of endogenous Pfr are probably not sufficient to significantly affect the COP1-SPA complex.

*FT*mRNA at dusk, but particularly during the night. These phenotypes are qualitatively very similar to those we observed for the phyB overexpressing lines. Therefore, we proposed that phyB Pfr accelerate flowering by partial inhibition of the function of the COP1-SPA complex. It has been demonstrated recently that the Pfr conformer of phyA and phyB binds to SPA1 disrupting the SPA1-COP1 interaction that results in lower activity of COP1 and accumulation of target proteins like HFR1 or HY5. Using yeast two-hybrid assays and light conditions where Pfr levels are limited, we showed that binding efficiency of SPA1 to the wild type or phospo-mutant derivatives of phyB tightly correlated with *FT* mRNA levels measured during the night in the transgenic lines overexpressing the corresponding phyB derivatives. This finding suggests that overexpressed phyB Pfr controls CO protein levels and flowering time by impairing SPA1-COP1 interaction. In contrast to the effect of overexpression, phyB Pfr in wild type plants promotes degradation of CO during the day. Since CO is ubiquitinated and degraded by the proteasome, phyB is expected to positively modulate the function of an ubiquitin ligase other than COP1 during the day.

6.In contrast to its role in flowering time determination, the effect of phyB on the pace of the clock in continuous red light is proportional to the amount of the protein: phyB mutants show long period phenotypes, whereas phyB overexpressors display shorter periods. According to the estimated levels of Pfr forms, phyB[S86A] and phyB[S86D] plants produced shorter and longer periods as compared with phyB[WT] plants at lower fluences of red light, but periods were identical in these lines under saturating illumination. Interestingly, periods in phyB[S86D] plants matched the periods in *phyB-9* plants at fluence rates lower than 35 µmol m^{-2} s^{-1}. In contrast, relative hypocotyl length in these two plants became identical at more than one order of magnitude lower fluences of red light.

RESULTS

1.phyB overexpression has been known to accelerate the clock (i.e. shortening periods) in a light dependent manner. We showed that this effect is specific for red light and does not result in early phasing of circadian rhythms in white light/dark cycles, where the early flowering phenotype is clearly detectable. Therefore, our results ruled out altered clock function as the potential cause of early flowering of phyB overexpressors.

2.In the next step, mRNA accumulation patterns of *CO* and *FT* were determined in SD and LD conditions. Overexpression of phyB had no effect on *CO* mRNA levels, but induced *FT* expression around dusk and during the night. The increase of *FT* mRNA in the phyB overexpressing lines, as compared to that in the wild type, was most dramatic in the night of SD conditions.Analysis of phyB-OX *ft-10* double mutant plants showed that the effect of phyB overexpression on flowering time was completely suppressed by the *ft-10* mutation. This finding strongly suggested that elevated expression of *FT* underlies the early flowering phenotype of phyB overexpressors. Interestingly, early flowering of *phyB* mutants is also mediated by increased *FT* transcription, but this effect is limited to the daytime; in contrast to the effect of phyB overexpression on *FT* mRNA levels that is detected around dusk and in the night.

Since CO is the main activator of *FT*, we created phyB-OX *co-9* double mutant plants to test if CO is required for the molecular and physiological flowering phenotypes of phyB overexpressors. Both early flowering and the induction of *FT*were diminished by the *co-9* mutation demonstrating that high levels of phyB up-regulate *FT* through CO. Since CO transcription was unaffected, we concluded that phyB overexpression enhances the function of CO at the post-translational level, most likely by stabilizing the CO protein.

3.In the dark, phytochromes are present in the inactive red light (λmax = 660 nm) absorbing form (Pr), which is converted to the biologically active far-red light (λmax = 730 nm) absorbing conformer (Pfr) upon red light irradiation. The active Pfr form is promptly and effectively converted back to the inactive Pr form by absorbing far-red light (photoconversion), or by a slower, light independent relaxation process called dark reversion. In wild type plants phyB facilitates degradation of CO in a red light dependent (i.e. Pfr-dependent) manner in the first half of the day. We employed parallel approaches to see if the effect of overexpressed phyB is also Pfr-dependent. First, we applied end-of-day far-red (EODFR) treatments in order to eliminate Pfr forms at the end of the photoperiod of a short day. The treatment diminished accumulation of *FT* mRNA in the night and significantly reduced the peak of *FT* expression at dusk, verifying that up-regulation of *FT* at these times was due to overexpressed phyB Pfr.

4.Second, we analyzed the molecular and physiological phenotypes of transgenic lines overexpressing mutant versions of phyB with conditionally or constitutively altered Pfr levels. Phosphorylation of phyB at Ser-86 has been shown to accelerate dark reversion of the receptor that affects signaling under non-saturating light conditions. In SD conditions, phyB[S86A] plants showed slightly higher induction of *FT* at night and flowered earlier than phyB[WT] plants. In contrast, phyB[S86D] plants displayed the same increase in *FT* mRNA levels at dusk as phyB[WT] and phyB[S86A] plants, but the peak of *FT* in the night was completely missing. Accordingly, phyB[S86D] plants flowered much later (44 leaves) than phyB[WT] (23 leaves) or phyB[S86A] (17 leaves), but earlier than Col wild type plants (54 leaves). These data indicate that induction of *FT* expression in the night plays the major role in early flowering of phyB overexpressors in SD conditions. In LD conditions, all phyB overexpressing plants displayed similar *FT* mRNA levels at dusk that were about 2-fold higher than that in Col plants. *FT*levels stayed high in

phyB[WT] and phyB[S86A] plants during the night, but dropped rapidly in phyB[S86D]. Since all phyB overexpressing lines flowered at the same time and earlier than Col plants, we concluded that elevated expression of *FT* at dusk is the main determinant of early flowering in LDs and persisting high levels of *FT* mRNA in the night do not contribute significantly to the phenotype. During the photoperiod of both SD and LD conditions plants were exposed to saturating fluences of light producing roughly equal amounts of Pfr by the end of the light phase in all lines that is reflected in very similar induction of *FT* around dusk. During the dark period of both SD and LD conditions, Pfr levels rapidly decreased in phyB[S86D] plants due to faster dark reversion. Depletion of Pfr was slower in phyB[WT] or even slower in phyB[S86A] plants. Accordingly, *FT* levels during the night were low in phyB[S86D], but high in phyB[WT] and phyB[S86A]. Taken together, the pattern of *FT* mRNA accumulation correlated well with the expected Pfr levels in the different lines and conditions.

phyB[Y276H] plants express phyB that constitutively exists in the Pfr form, whereas phyB[C357T] plants produce phyB Pr independent of the light conditions due to impaired binding of the chromophore. phyB[Y276H] plants showed *FT* expression profiles and flowering time identical to those of phyB[S86A] plants, whereas phyB[C357T] plants behaved as the *phyB-9* mutant corroborating the requirement of phyB Pfr for the flowering phenotype of phyB overexpression.

5.Our data strongly suggested that high levels of phyB Pfr stabilize the CO protein around dusk and during the night. The COP1-SPA ubiquitin ligase complex was shown to play the major role in the regulation of CO protein levels at these times. The four SPA proteins (SPA1-SPA4) redundantly enhance the ubiquitin ligase activity of COP1 via physical interactions. SPA1 and SPA4 were shown to be the primary SPA proteins controlling flowering time. The *cop1* and *spa* mutants flower early especially in SD conditions and have increased levels of CO protein and