Thesis of Ph.D. dissertation

Analysis of regulatory function of circadian clock on photoreceptor gene expression

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INTRODUCTION

During their entire life cycle plants are very sensitive to their light environment. Light is a key factor influencing all major developmental transitions from seed germination to induction of flowering. Plants accurately perceive fluctuations in the intensity, spectral quality, directionality, and periodicity (day length) of the incoming light. Plants possess photoreceptors: UV-A and blue light absorbing cryptochromes (cry) and phototropins (phot), red and far-red light absorbing phytochromes (phy).

In *Arabidopsis thaliana*, phytochromes are encoded by a small multigene family, *PHYA* through *PHYE* (Sharrock and Quail, 1989; Clack et al., 1994). Phytochromes have a covalently linked chromophore, a linear tetrapyrrole. These photoreceptors exist in two interchangeable spectral forms: the inactive P_r form (red light absorbing) phototransforms into the active P_{fr} form (far-red light absorbing) upon absorption of red light. This reaction can be reversed when P_{fr} is converted to P_r upon absorption of far-red light (Furuya, 1993). phyA is a photolabile molecule degrading rapidly when exposed 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. These molecules mediate responses to low and high fluences of red light (Furuya and Schäfer, 1996).

Plant cryptochromes have sequence homology to distinct types of photolyases in bacteria and animals, but lack photolyase activity and possess a distinctive C-terminal extension (Cashmore et al, 1999). They bind two chromophores, a pterin and FAD. Cryptochromes are clearly important for normal control of growth and development induced by blue light. cry2 is particularly important in the response to low blue light intensities and cry1 has a prevalent role in the response to strong blue light (Lin, 2002). In contrast to cry2, which is strongly down-regulated in response to blue light, cry1 protein levels are not light-regulated (Cashmore et al. 1999; Lin et al, 1998). This presumably explains why cry2 plays a major role in low light intensities and cry1 in high light intensities.

Many light effects are induced by the co-action of several photoreceptors and most photoreceptors regulate multiple aspects of photomorphogenesis by forming an interacting network (Casal, 2000).

Many biological organisms possess internal molecular timekeeper mechanisms, circadian clocks, allowing the anticipation of regular fluctuations in the availability of the most important resources, such as sunlight. The clock imposes a 24-hour rhythm on certain physiological processes so that they always occur at the optimal phase of the light–dark cycle. They range from leaf movement, growth processes and flower opening to photosynthesis and carbon metabolism (Harmer et al, 2000). Underlying many of these physiological rhythms there are endogenous rhythms in gene activity.

The circadian system can be divided into three main parts (Somers, 1999). 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. The oscillator regulates the expression of genes through the output pathway. For their proper function, the clocks are synchronized to the periodic environmental changes (e.g. day/night cycles) by specific stimuli. The entraining signals are transduced by input pathways. According to the classical model of circadian systems there is a one-way relationship between the input elements and the oscillator without feedback mechanisms.

For higher plants the most important entraining environmental factor is light. In Arabidopsis the light input pathway involves several photoreceptors. Both phytochromes (phyA, B, D, E) and cryptochromes (CRY1, 2) have been proven to play a role in setting the clock and maintaining the proper period length by transducing the light signal to the central oscillator (Somers et al., 1998a; Devlin and Kay, 2000). Mutations on phytochrome (*PHYA* and *PHYB*) and cryptochrome (*CRY1* and *CRY2)* genes increase the period of rhythmic expression of a photosynthetic gene (CAB2-chlorophyl a/b binding protein 2) under certain fluence rates of red or blue light (Devlin and Kay, 2000; Somers et al., 1998a). The quadruple *phyAphyBcry1cry2* mutant still retains rhythmicity in *Arabidopsis*, indicating that these photoreceptors are not essential components of the clock (Yanovsky et al., 2000). Thus until now the photoreceptors have been considered as being elements only of the light input pathways.

RESEARCH OBJECTIVES

The expression of photoreceptor genes was demonstrated to be regulated by light (Somers and Quail, 1995a, 1995b; Goosey et al., 1997), but the effects of other endogenous or enviromental factors have been unknown. Our group previously reported that the Arabidopsis circadian clock controls the expression of the phyB photoreceptor (Kozma-Bognár et al., 1999). Light input is mediated by multiple photoreceptors, it is unclear, however, how many of these photoreceptors are regulated by the clock.

The general goal of our research was to perform a detailed comparative analysis of the spatial, temporal, and long-term expression patterns of all phytochrome and cryptochrome genes in Arabidopsis with respect to their circadian regulation.

The main objectives of this work were:

1. To compare the spatial expression patterns of phytochromes and cryptochromes in Arabidopsis seedlings.
2. To determine whether the expression of phytochromes and cryptochromes is regulated by the circadian clock like that of *PHYB* and, if the answer is yes, at what level(s) of expression.
3. If their expression is rhythmic, to investigate the tissue- and age-specificity of the circadian expression of one selected photoreceptor.

METHODS

- Culturing *Arabidopsis thaliana* plants under sterile and greenhouse conditions
- Generation of transgenic plants
- Molecular cloning techniques
- Plant total RNA extraction
- Northern-blotting, RNase protection assay
- *In vivo* luciferase enzyme activity measurements

RESULTS AND DISCUSSION

1. Transgenic Arabidopsis plants expressing the luciferase gene (*LUC+*) under the control of photoreceptor promoters were constructed. In these transgenic plants luciferase enzyme activity reflects the activity of the photoreceptor promoter to which LUC+ is fused. On this basis the promoter-luciferase system allows the identification of spatial patterns of expression, providing a possibility for comparative analysis of their tissue-specific promoter activities. In light-grown seedlings high promoter activities were detected for *PHY*A and *CRY2* in shoot meristems and root tips, and lower activities in cotyledons, hypocotyls and roots. *CRY1* were expressed in aerial tissues; *PHYB* in all tissues, in particular in shoot meristems and root tips, whereas *PHYC*, *PHYD* and *PHYE* were expressed in cotyledons and root tips. In etiolated seedlings *CRY1* expression was restricted to cotyledons and the upper part of the hypocotyl. This pattern supplemented with a weak activity in root tips was also characteristic for *PHYB*, *PHYC* and *CRY2* genes. The promoter activity for *PHYC* was most dominant in the folded cotyledons, in contrast to that for *PHYA* and *PHYE,* detected mostly in the hypocotyl. These results revealed new details of the tissue-specific expression and light regulation of the *PHYC* and *CRY1* and *CRY2* promoters.

2.1 Seedlings carrying the various promoter::LUC+ reporter constructs were entrained in 12h white light/12h dark (LD) cycles and were subsequently imaged under the same conditions. By detecting luciferase activity in the transgenic plants we demonstrated that the genes encoding the major photoreceptors are not uniformly active throughout the day. Rather, the promoter activities of phytochromes and cryptochromes is diurnally regulated in light/dark cycle, with peaks of expression at different time points during the light period.

One of the hallmarks of circadian rhythms is their persistence under constant conditions. In order to determine whether the diurnal rhythm of the promoter activity of photoreceptors was due to the regulatory effect of the circadian clock, LD-grown seedlings were transferred to circadian conditions: continuous light (LL) or continuous dark (DD). Upon transfer to constant conditions (light or dark), circadian regulation is maintained for all genes, with the exception of *PHYC*, indicating that – besides the effect of light - the circadian clock controls the transcription of the photoreceptor genes.

2.2 LD-grown wild-type seedlings were shifted to LL conditions. Total RNA was isolated from samples harvested in 4-h-intervals and the abundance of phytochrome and cryptochrome mRNA molecules was determined by Northern hybridization or RNase protection. In accordance with the results of luciferase activity, the mRNA accumulation of most photoreceptor genes followed the rhythmic pattern in similar phase. The only exception was *PHYC* gene, whose mRNA level displayed clear circadian oscillations unlike its promoter activity. This fact indicates that the circadian regulation of *PHYC* gene expression is exerted at the level of mRNA accumulation rather than at the level of promoter activity. Transcripts encoding the light-stable proteins phyB, phyC, phyD, phyE and cry1 peak during the early hours of the daily light period, whereas those encoding the light-labile phyA and cry2 reach their highest level close to dusk. The phase of rhythms reflects the importance of the photoreceptors for different light conditions.

3. We selected *PHYA* gene expression for the detailed analysis, because the function of phyA among the phytochromes is non-redundant, due to its light-labile nature. The expression pattern of *PHYA::LUC+* was compared in seedlings and adult plants under DD conditions. *PHYA* expression was robust in seedlings, but dampened rapidly in adult plants, indicating that circadian regulation is altered at different developmental stages. To investigate tissue-specific effects, the PHYA expression rhythm was determined in intact and excised organs of adult plants. High-amplitude rhythms were maintained for many days in isolated leaves in darkness, whereas the leaves of intact plants rapidly lost rhythmicity. The wounding of the leaves of intact plants and callus formation had no effect on rhythmic expression. In excised roots and hypocotyls, the oscillation of *PHYA* expression was different from that in leaves, indicating the existence of multiple factors responsible for organ-specific regulation and systemic regulation of the circadian clock.

The amplitude and damping of *PHYA* expression rhythms in light-grown Arabidopsis plants are variable, depending on the type and the age of tissue tested. The rhythmic pattern of *PHYA* expression is not organ-autonomous but depends on the physical continuity, consistent with the presence of a transmitted signal that controls the overt expression of circadian rhythms without necessarily affecting the underlying clock.

CONCLUSION

The circadian clock regulates the expression of all phytochromes and cryptochormes at both the levels of promoter activity and mRNA abundance with different efficiencies. The expression of photoreceptor promoters is controlled in a plastic manner by a network of endogenous clock and light, which presumably contributes to the adaptive regulation of light perception and light signalling.

The tissue-specific differences in photoreceptor gene expression could therefore contribute to the differential light responses of various tissues, including even differential regulation of circadian system. A circadian system might be present in most, if not all, plant cells, but its effect on intracellular rhythms can be controlled by supracellular signalling.

Photoreceptors, on the one hand, transduce light signals to the clock and thus are part of the input pathways on the other hand, they also have to be considered part of the clock output. The rhythmically produced photoreceptors can temporally restrict light input to the clock by feedback mechanism, so that resetting cues work most efficiently at the appropriate times of day.

Furthermore, photoreceptor genes can be used as new molecular markers to study circadian regulated gene expression in Arabidopsis, and the *PHY* and *CRY* genes form a special subgroup of the circadian markers, because they can be placed in the input as well as the output pathways.

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LIST OF PUBLICATIONS

For the dissertation the 2. and 3. publications has been used.

1. Kim, L., Kircher, S., **Tóth, R**., Ádam, É., Schäfer, E., Nagy, F. (2000) Light-induced nuclear import of phytochrome-A:GFP fusion proteins is differentially regulated in transgenic tobacco and Arabidopsis. *Plant J,* 22:125-33.

2. **Tóth, R**., Kevei, É., Hall, A., Millar, A., Nagy, F., Kozma-Bognár, L. (2001) Circadian clock-regulated expression of phytochrome and cryptochrome genes *Arabidopsis*. *Plant Physiol*, 127:1607-1616.

3. Hall, A., Kozma-Bognár, L.**, Tóth, R**., Nagy, F., Millar, A. (2001) Conditional circadian regulation of *PHYTOCHROME A* gene expression. *Plant Physiol*, 127:1808-1818.