Thesis of Ph.D. dissertation

INTERACTION OF THE PLANT CIRCADIAN CLOCK AND UV-B SIGNALLING PATHWAYS

Balázs Fehér

Supervisor: Dr. Ferenc Nagy

School of Biology, University of Szeged

Biological Research Centre of H.A.S.

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INTRODUCTION

Circadian clocks are ubiquitous biochemical timing mechanisms that rhythmically regulate a wide range of molecular and physiological processes in most of the living organisms exposed to the daily succession of days and nights (Harmer, 2009). Due to the function of the clock, certain processes are timed to the most appropriate time of the day and are attenuated, when they are not needed. This temporal organization results in significant saving of chemical energy and contributes to the fitness of the organism (Dodd et al., 2005).

In *Arabidopsis* the central oscillator relies on the function of three regulatory circuits (Locke et al., 2006). The "morning loop" consists of the Myb-related CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and the LATE ELONGATED HYPOCOTYL (LHY) transcription factors, which facilitate the transcription of the *PSEUDO RESPONSE REGULATOR 7/9* (*PRR7/9*) genes in the morning. PRR7/9 proteins inhibit transcription of *CCA1*/*LHY* during the day (Farré et al., 2005). The evening loop is formed by TIMING OF CAB EXPRESSION 1 (TOC1) and GIGANTEA (GI). GI positively regulates *TOC1* expression in the evening, whereas TOC1 attenuates GI expression during the night. The morning and evening loops are coupled by a third circuit, where TOC1 indirectly induces transcription of *CCA1*/*LHY* during the late night and CCA1/LHY inhibit *TOC1* expression in the morning (Alabadi et al., 2001). The coordinated function of these three circuits is required to generate the basic oscillation in the level of the clock.

The biological impact of the clock depends on the synchrony between the subjective time provided by the oscillator and the real time of the environment. To achieve this, the phase of the oscillator is reset to the day/night cycles by periodic environmental signals among which light is the

most significant. Light is perceived by photoreceptors and signals are transduced to the oscillator via the light input pathway, where they affect the level and/or activity the clock components.

UV-B light has long been known as a harmful component of the sunlight causing damage to DNA, protein and other macromolecules capable of direct absorption of these wavelengths of light (Jansen et al., 1998). However, UV-B at lower fluence rates has been shown to act as an environmental signal to control development, promote photomorphogenesis and drive the expression of genes required for the production of flavonoids (Ulm et al., 2004). Although the photoreceptor, which is thought to mediate all of the UV-B effects, is still unknown, several components of the signaling cascade have been identified recently. UV RESISTANCE LOCUS 8 (UVR8) is a protein required for virtually all physiological UV-B responses, indicating that it is located very close to the putative receptor in the signaling cascade (Favory et al., 2009) or it is the low fluence UV-B photoreceptor itself (Rizzini et al., 2011). UVR8 is highly specialized for UV-B signal transduction since *uvr8* mutants show UV-B-dependent phenotypes only (Brown et al., 2005). The E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) physically interacts with UVR8 in a UV-B dependent manner and this interaction is suggested to be required for signaling (Favory et al., 2009). Under supplementary UV-B, COP1 promotes transcription of the *ELONGATED HYPOCOTYL 5 (HY5)* gene. HY5 and HY5 HOMOLOG (HYH) act as transcription factors and play crucial roles in mediating molecular and physiological processes in response to photomorphogenic UV-B light (Brown and Jenkins, 2008). Low intensity UV-B light induces the expression of genes implicated in flavonoid biosynthesis. In turn, accumulation of UV-B absorbing flavonoids confers protection to plants

against the damaging effects of high intensity UV-B, which is apparent under natural conditions.

RESEARCH OBJECTIVES

Entrainment is required to set and maintain the correct and stable phase-relationship between the endogenous circadian clock and environmental light/dark cycle. Since UV-B is an important component of sunlight, we ought to test if low intensity, non-damaging UV-B contributes to the light-mediated entrainment of the circadian clock in Arabidopsis.

Most biochemical processes are organized by the circadian clock. This timing results in saving of resources and improves fitness. Therefore we want to know, how does the circadian clock regulate the UV-B responsive processes.

RESEARCH METHODS

- Molecular cloning techniques
- Creation and maintenance of transgenic *Arabidopsis thaliana* plants
- *In vivo* luciferase enzyme activity measurements in intact seedlings
- Determination of period length of circadian rhytms by using BRASS software
- Plant genomic DNA extraction
- Plant total RNA extraction
- Quantitative Real-Time PCR assay
- Total plant protein isolation, Western-blotting

RESULTS

The function of the light input pathway affects the pace or the phase of the oscillator, depending on the light conditions. In plants, continuous irradiation

shortens the free-running period length in a fluence rate dependent manner. To test the effect of UV-B in this process, WT, *uvr8-6, cop1-4,* and *hy5 hyh* mutant plants expressing the *CCR2:LUC+* reporter were grown in 12/12 LD cycles for a week and transferred to continuous white light (WLL) or WLL supplemented by UV-B of different fluence rates. Rhythmic *CCR2:LUC+* expression was monitored and free-running periods were calculated. UV-B shortens the period in WT and in *hy5 hyh* mutant a fluence rate dependent manner, but not in *uvr8-6* and in *cop1-4* mutants. The effect of the *uvr8-6* mutation is specific for UV-B light, since it does not affect period length in plants free-running in WLL or in darkness.

In contrast to the effect of continuous irradiation, discrete light pulses elicit characteristic phase shifts of the free-running oscillator. Phase response curves (PRCs) are constructed by plotting the magnitude of the phase change against the circadian time when the light pulse was administered. It has been demonstrated that red or blue light pulses given during the early or late subjective night trigger phase delays (negative phase changes) or phase advances (positive phase changes), respectively. To test the effect of UV-B in phase re-setting, plants described above were grown in 12/12 LD cycles for a week and transferred to WLL. Short UV-B pulses (1.5 μmol m^{-2} s^{-1} for 30 min) were given to parallel sets of plants in every 3 hr starting at 36 hr after the transfer to WLL. The magnitude and direction of phase shifts of *CCR2:LUC+* expression were determined relative to the non-pulsed control plants. UV-B pulses cause phase delays during the subjective night, but apparently have no effect during the subjective day in WT plants. In contrast, no significant phase shifts were detected in *uvr8-6* or in *cop1-4* mutant plants at any time of the circadian cycle. On the other hand, when white light pulses were used to induce phase shifts in plants free-running in DD, WT and *uvr8-6* plants displayed identical PRCs with the shape consistent with published

data (Covington et al., 2001). Collectively, these data demonstrate that UV-B light does entrain the plant circadian clock, and UVR8 and COP1 play essential roles in this process.

The precise molecular mechanism by which light signals entrain the plant circadian oscillator is not fully understood yet, but light-modulated transcription of core oscillator genes is thought to contribute to this process. In Arabidopsis, transcription of clock genes *CCA1*, *LHY*, *PRR9 GI* and the clock-associated gene *ELF4* is acutely induced by visible light. Induction of these rhythmically expressed genes is gated by the clock, which means that the same light treatment causes maximal induction at the time around the circadian peak of a given gene, but only a small increase is detected at the time of the circadian trough.

To see if UV-B affects the transcription of clock genes in Arabidopsis, the acute induction of *CCA1*, *PRR9*, *GI* and *ELF4* genes was tested by quantitative RT-PCR. WT, *uvr8-6* and *cop1-4* plants were grown in 12/12 LD cycles for a week and transferred to WLL. Plants were irradiated by UV-B (1.5 μmol m^{-2} s^{-1} for 30 min) at 3 hr intervals starting from 36 hr after the transfer, returned to WLL and harvested 1 hr later. The control, non-pulsed samples were harvested at 3 hr intervals and provided data on the basal expression of the corresponding genes. *CCA1*, *PRR9*, *GI* and *ELF4* showed acute and gated induction in response to UV-B pulses. The *uvr8-6* mutation had no effect on the basal circadian expression of these genes, but eliminated the response to UV-B pulses. In contrast, the early phase and short period phenotype of the *cop1-4* mutation was clearly seen in the case of all clock genes tested and UV-B induction of all tested was diminished. Expression of the clock gene *TOC1* is not affected by visible light, and *TOC1* is not affected by UV-B light either. These data suggest that UV-B induces the expression of a very similar set of clock genes as visible light and

demonstrate that UVR8 regulates clock gene expression in response to UV-B, but not visible light.

The circadian clock rhythmically attenuates visible light responses (Hotta et al., 2007). To see if UV-B responses are influenced similarly, UV-B induced circadian genes (*CHALCONE SYNTHASE* (*CHS, At5g13930*), *GLUTATHIONE PEROXIDASE 7* (*GPX7, At4g31870*), *EARLY LIGHT INDUCED PROTEIN 2* (*ELIP2, At4g14690*), *ELONGATED HYPOCOTYL 5* (*HY5, At5g11260*) and *HY5-HOMOLOG* (*HYH, At3g17609*)) and non-circadian genes (*At3g16350* and *At3g57830*) were selected and their UV-B inducibility was tested in WT, *uvr8-6* and *cop1-4* plants. Induction of *CHS*, *GPX7*, *ELIP2* and *HYH* showed clear circadian control with amplitude comparable to that of the clock genes. In contrast, inducibility of *HY5* did not display any circadian patterns. To see if the lack of gating is general for any light responses of HY5, the effect of red light pulses was tested in a "classical" gating experiment. Plants were grown in 12/12 LD cycles for a week and transferred to DD. Red light pulses (40 μmol m^{-2} s^{-1} for 60 min) were given at 3 hr intervals and *HY5* and *HYH* mRNA levels were determined in pulsed and nonpulsed samples. It seems, red light induction of *HY5* and *HYH* is gated by the clock.

Interestingly, UV-B induction of the non-rhythmic *At3g16350* and *At3g57830* genes showed clear circadian modulation, and the maximal induction was detected around mid-day. To see if the low expression level of these genes in the absence of UV-B masks circadian rhythmicity, we measured transcript levels in plants transferred to WLL supplemented with UV-B. We conclude, despite the elevated levels under these conditions, these genes are expressed non-rhythmically.

UV-B induction of all genes tested was completely eliminated in *uvr8-6* or *cop1-4* plants. The *uvr8-6* mutation had no effects on basal expression

levels/patterns, but *cop1-4* caused an early phase/short period phenotype as expected. Moreover, the basal expression level of *CHS*, *ELIP2* and *HY5* was elevated in *cop1-4* plants. Taken together, these data suggest that -at least gene expression- gating of UV-B and visible light induction is differently regulated.

The HY5 and HYH transcription factors were suggested to control the UV-B induction of most responsive genes in Arabidopsis (Brown and Jenkins, 2008). To test their function in our conditions and for the particular target genes used in this study, a UV-B gating experiment was performed using *hy5 hyh* double mutant plants. The growth and induction conditions were essentially the same as described in the previous sections. In double mutant plants the basal expression level and UV-B induction of *CCA1* and *PRR9* was only slightly affected, whereas gating was clearly retained. In contrast, expression of *CHS* was severely reduced and UV-B induction was completely lost in *hy5 hyh*, as described before (Brown and Jenkins, 2008). In the case of *At3g16350* UV-B induction was diminished, but not completely lost. Importantly, the retained inducibility showed clear circadian gating. Collectively, these results demonstrate that UV-B induction of clock genes does not rely on HY5/HYH and circadian modulation of UV-B induced gene expression is not mediated by these transcription factors.

Circadian modulation of UV-B induction is provided by the circadian clock. In principle, if clock regulation is eliminated, gating is lost, but UV-B inducibility should be retained at a certain constant level. To test this, UV-B induced gene expression was investigated in plants with non-functional circadian clocks. *CCA1* showed low and constitutive basal expression level, whereas *PRR9* was expressed at high and constitutive level in non-induced *elf3-4* plants, in agreement with published data (Alabadi et al., 2001). The clock-controlled *CHS* and the non-rhythmic *At3g16350* showed increased

basal expression levels in *elf3-4,* similarly to *PRR9*. UV-B induction of all tested genes displayed constitutive, non-gated pattern in *elf3-4*. The induced mRNA levels of *PRR9*, *GI*, *CHS* and *At3g16350* in *elf3-4* were similar to or higher than the maximal induced levels in WT.

Analysis of the arrhythmic line constitutively over-expressing *CCA1* (CCA1-OX, line 38), (Wang and Tobin, 1998) yielded similar results. UV-B irradiation resulted in high levels of gene induction independent of the time of the treatment. Interestingly, two-fold induction of *CCA1* was observed in the CCA1-OX line. Since the 35S promoter is not up-regulated by UV-B (Boyko et al., 2010), this result suggests an effect of UV-B on the stability of *CCA1* mRNA. These data indicate that rhythmic gating is lost, but UV-B induction of gene expression is retained in these arrhythmic lines.

The observed hypersensitivity of UV-B dependent gene expression has suggested that *elf3-4* mutant plants could be more tolerant to UV-B stress compared to WT. To test this hypothesis, plants were grown in 12/12 LD cycles for a week, transferred to continuous white light supplemented with UV-B (1.5 μmol m^{-2} s^{-1}) and were given high-intensity (14 μmol m^{-2} s^{-1}) UV-B pulse for 20 min in the subjective morning and in the subjective evening. Plants were returned to normal growth conditions and analyzed one week later. WT and *elf3-4* plants were similarly affected by the UV-B pulse, indicating that the particular high induced levels of genes dependent on low intensity UV-B do not confer resistance to *elf3-4* plants against high intensity UV-B. Moreover, the effect of stressing UV-B pulses was independent of the time of the day in both genotypes.

DISCUSSION

Entraining light signals affect different parameters of the free-running clock, depending on the duration of irradiation. In plants, continuous irradiation

shortens the free-running period length in a fluence rate dependent manner, whereas light pulses elicit discrete phase shifts of the oscillator. Our data demonstrate that UV-B acts as a signal for both modes of entrainment. In WT seedlings the clock output markers exhibited significantly (1-2 h) shorter free-running periods if continuous white light was supplemented by low fluences of UV-B. UV-B pulses applied to plants free-running in WLL were effective in causing phase delays during the night, but no phase shifts were elicited during the day.

To provide a possible molecular mechanism for UV-B mediated entrainment, the inducibility of the core clock genes *CCA1*, *PRR9*, *GI* and the clock-associated gene *ELF4* was tested over one circadian cycle. All genes showed rhythmically gated UV-B induction in WT seedlings and that their induction was eliminated in *uvr8-6* and *cop1-4* plants. Surprisingly, the loss of HY5/HYH did not affect the period length of the clock in WL or in UV-B and the UV-B inducibility of clock genes. These findings are somewhat unexpected as HY5 was shown (i) to bind *in vivo* to the promoters of several clock genes, including *CCA1*, *LHY*, *ELF4* and *TOC1* in seedlings grown in WL (Lee et al., 2007), (ii) the *hy5* and *hy5 hyh* double mutant, similarly to *uvr8*, is impaired in UV-B dependent photomorphogenesis, (Oravecz et al., 2006) and (iii) *hy5* was thought to mediate UV-B inducibility of the majority of genes regulated by UVR8 and COP1 (Brown et al., 2005).

Taken together, the correlation of physiological (modulation of period length) and molecular (clock gene induction) data from UV-B signaling mutants suggests that this effect of UV-B is mediated through the transcriptional regulation of clock genes. Among the clock genes tested, *PRR9* showed the most significant induction to UV-B. The elevated level of *PRR9* in continuous UV-B light could explain the effect of UV-B on period

length, since over-expression of *PRR9* resulted in a similar degree of period shortening (Matsushika et al., 2002).

Circadian gating is the process by which the clock rhythmically inhibits the acute effect of environmental or endogenous signals on the corresponding target processes. UV-B induction of all but one tested clock and clock-controlled genes, was gated by the clock in a manner similar to gating visible light responses. The only, but significant exception was *HY5*, which showed a flat, non-gated induction by UV-B but a gated induction by red light. These results demonstrate that the signaling pathway mediating the UV-B induction of *HY5* is not targeted by the clock and it is different from the cascades transducing visible light signals to *HY5*.

Unexpectedly, UV-B induction of the non-rhythmic *At3g16350* and *At3g57830* showed clear circadian gating with peak levels of induction at the middle of the subjective day. Gating is not restricted to a single time of day, but works for a range of genes with markedly different phases of expression. Therefore the mechanism underlying gating could be explained by UV-B signaling components acting in parallel routes and controlled by the clock in different phases. *UVR8* is transcribed rhythmically and shows the peak of expression close to dusk. However, UVR8 protein levels show no daily oscillations, so it is unlikely that this mechanism mediates gating of UV-B induction, although the possibility of clock-regulated post-translational modification of UVR8 cannot be excluded. *HY5* and *HYH* show a low amplitude rhythm in WL but UV-B induction of *HY5* lacks clock control whereas the UV-B induction of *HYH* is gated; therefore, accumulation of HY5/HYH heterodimers in the morning could mediate gating of UV-B responses of morning expressed genes. However, our data demonstrate that clock gene induction by UV-B is unaffected in the *hy5 hyh* double mutant in the morning (*CCA1*) or during the day (*PRR9*). UV-B induction of *CHS* was

completely absent, but a residual and gated induction of *At3g16350* was retained in *hy5 hyh*, indicating that HY5/HYH are important for the acute UV-B induction of a set of genes, but they are dispensable for the temporal modulation of the response.

Induction of gene expression in response to low fluences of UV-B light (acclimation) has an important function in triggering the production of pigments (flavonoids, anthocyanin) that are protective against high intensity, damaging UV-B (Favory et al., 2009). Our results showed that induction of all tested non-clock genes was gated to the subjective morning or the first part of the subjective day. This indicated that perception and processing of low intensity UV-B signals in the early part of the day is important to set up the flavonoid "shield" and survive UV-B stress expected during the subsequent hours of the day. In order to test the physiological relevance of clock modulated induction of gene expression in response to low intensity UV-B, we characterised molecular and physiological UV-B responses in the arrhythmic *elf3-4* mutant. Our data demonstrated that almost all of the genes tested here showed a high induction level to low fluences of UV-B in *elf3-4* at all times of the day. The induced mRNA levels, except for *CCA1*, were usually higher than the maximal induction level achieved in WT which in turn suggested that *elf3-4* plants may display increased stress tolerance to UV-B compared to WT plants. However, acclimated *elf3-4* mutants were as resistant to UV-B stress pulses applied at different times of the day as were the acclimated WT controls. Analysis of another arrhythmic mutant, CCA1-OX, has provided similar results. This indicates that limiting low intensity UV-B dependent gene expression and the subsequent accumulation of flavonoids to a relatively short interval of the day is sufficient to reach normal protection against the damaging effect of high intensity UV-B

LIST OF PUBLICATIONS

Publication used in the thesis:

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