Study on the molecular mechanisms for high-light acclimation in the green alga *Chlamydomonas reinhardtii*

Yari Kamrani, Yousef [1,2]

[1]Department of Basic Biology, School of Life Science, Graduate University for Advanced Studies (SOKENDAI), Okazaki, Aichi, 444-8585 Japan

[2]Division of Environmental Photobiology, National Institute for Basic Biology, Okazaki, Aichi, 444-8585 Japan

Introduction: *Chlamydomonas reinhardtii* is a suitable model organism for studying responses to excess light due to it being easily grown in liquid or on agar in a wide range of environmental conditions. The cells of *C. reinhardtii* have a single chloroplast and have photosynthetic machinery that is very much like that of vascular plants. Moreover, *C. reinhardtii* is easy to grow and can be exposed to different light intensities in an even manner. However, excessive light can be harmful to photosynthetic organisms despite the fact that it is required for their growth in most cases. Because of this *C. reinhardtii* has acquired several ways to lessen light stress to defend against and repair damage caused by an overabundance of light. Photoprotective mechanisms exist for quenching excess ^{1}Chl* and dissipating the energy harmlessly as heat when light intensity is higher than the tolerance of the photosynthetic machinery. These mechanisms are measured from the reduction of Chl fluorescence from PSII and are referred to as non-photochemical quenching (NPQ). The most rapid type of NPQ is energy-dependent quenching qE. LHCSR3 is required for qE. However, regulation of *LHCSR3* expression remains to be investigated. Since the majority of photoacclimation analyses has been conducted under controlled laboratory conditions, physiological responses to natural environmental changes are not well clarified; in plants and microalgae, light-dark cycles are required to synchronize circadian clocks to multiple physiological responses. However, clock response to high light has been the subject of speculation. On the other hand, very recent research has shown blue light

perceived by PHOT (phototropin photoreceptor) mediates the photoprotection of the photosynthetic machinery and regulation of LHCSR3 in *C. reinhardtii*. The same research suggested as PHOT regulate LHCSR3 expression in *C. reinhardtii* and Ca^{2+} is required for the accumulation of LHCSR3, a relationship between PHOT, Ca^{2+}, and LHCSR3 is likely. The aim of my research was to determine how these mechanisms are involved and coordinated in the regulation of *LHCSR3* gene under high-light acclimation in *C. reinhardtii*. Our methods were basically relied on two strategies. First one was to investigate the impact of the circadian clock on regulation of *LHCSR3*. Previously, 105 circadian rhythm insertional mutants were isolated as *rhythm of chloroplast* (*roc*) mutants. Due to the versatile function of ROC series mutants in *Chlamydomonas*, we focus mainly on characterization of these clock mutants with respect to qE value and LHCSR3 expression under strong blue and red light. To proceed with second strategy, we established and developed techniques to visualize cytosolic Ca^{2+} in *Chlamydomonas* and use this approach to investigate those molecular mechanisms which are involved downstream of PHOT and ETR to regulate *LHCSR3* expression. Following, to explore our achievement in calcium signaling, a high throughput forward genetic approach was used to screen mutants under strong blue light using bioluminescence derived from a luciferase reporter gene fused to a full-length sequence of *LHCSR3* gene. This screening strategy was used to pinpoint more important factors and elements involved in blue-light- dependent regulation of *LHCSR3* gene expression.

Methods:

Strains and conditions

C. reinhardtii strains, CBR34(mt+), SAG73.72 (mt+) and CC-125 (137c mt+) were used as WT control. They were grown in flasks with orbital shaking in Tris-acetate-phosphate medium under 15 µmol photons $m^{-2}.s^{-1}$ white light unless otherwise stated. At 3 million cells ml^{-1}, conditions were switched to HS minimal medium, and the cells were irradiated with different light intensities. The roc series mutants were isolated and identified as described previously by Matsuo et al. 2008. *pcry* and *phot* (Zorin et al., 2009), mutants were used for quantitative PCR analysis. For continuous-dark treatments, culture aliquots were transferred to flasks wrapped with aluminum foil. Light intensities were measured at the surface of plates using a silicon photodiode-based photon meter (QTM-101. Monotech Inc., Saitama; Japan).

Chlamydomonas cells were entrained for three days under 12 h LD cycles for quantitative PCR analysis of the *ROC75* transcript. The 12 h light period was divided in three section (3:6:3 h red: red+green+blue: red, respectively) to more closely mimic the spectral fingerprint of natural light at dawn, noon and dusk. The photoperiodic regime was formed of red (660 nm), green (550 nm) and blue (450 nm) LEDs using 100 µmol photons $m^{-2}.s^{-1}$ equally of each LED light. For dark samples, cells were recovered at the end of the 12 h of dark period of the LD regime. For HL samples, cells were exposed to blue light at 250 µmol photons $m^{-2}.s^{-1}$ for 1h at the end of the 12 h of dark period of the LD regime.

Construction of the *LHCSR3.1-pLUC* reporter gene

aphVIII as a selection marker was amplified from pRT-GenD-CrmVenus_aphVIII vector as template by PCR with the primer sets aphVIII-F1 (5′- gcctctttctccatgctcgagcggggagctcgctg-3′)/ aphVIII-R1 (5′- attacgccaagcttgggtacccgcttcaaatacgcc-3′) and inserted into linearized pHSG/lucNCi-TRBCS2 vector (*NcoI, sphI*) gifted form Dr. Takuya Matsuo-Nagoya university. LHCSR3.1 promoter was amplified from genomic DNA by PCR with the primer sets *LHCSR3.1*-P-F1(FW:5'-TCTCGAATTCGCTGACTCCCCTGTCTTCAG-3')/*LHCSR3.1*-P-R1(5'-TCGCTTTAAATGTGAGTGCAAGTGGCGTGCAA-3') 1500bp upstream of ATG start codon to cover promoter region. To construct *LHCSR3.1* translational fusion, luciferase gene from pCR 2.1 TOPO/luc tag vector was cut using ClaI and this fragment ligated to linearized pRT-GenD-LHCSR3.1 (+Cla)_aphVIII with ClaI restriction enzyme. LHCSR3.1 in this plasmid was entire gene fragment including exons and introns. Plasmid were digested afterwards to verify the presence of the insert (luciferase) and for determining its orientation in the entry vector.

Results: As a first step I characterized 105 ROC series mutants by qE value under strong blue and red light and I report *ROC75*, a putative transcriptional factor as a key component of the central circadian clock, which showed a significantly higher qE value and LHCSR3 protein accumulation than the wild type when grown under red light. Further, *LHCSR3* mRNA in *roc75* mutant exhibited a circadian rhythm, with its basal expression level higher than in the wild-type. I therefore conclude that ROC75 acts as an attenuator for the circadian clock that controls *LHCSR3* expression with red light as a negative stimulus.

For the second step, by performing calcium imaging recordings, we noted that the basal cytosolic Ca^{2+} level in *Chlamydomonas* can be increased after 1-hour illumination with blue light (30

μmol·m^{-2} ·s^{-1}) in comparison with WT sample maintained in the dark, and this elevation was not observed in *phot* mutant. Additionally, I could elucidate the role of several key factors present in calcium signaling pathway by which PHOT regulates *LHCSR3.1* in *C. reinhardtii*. To proceed further with step two, we measured the bioluminescence of a reporter strain (*LHCSR3.1-lucCP*) that expresses the luciferase gene under the control of the *LHCSR3.1* promoter under high-throughput assay conditions developed for this study. However, we failed to detect significant luciferase activity in all 2000 transformants. One of the possible problems could be an epigenetic silencing of transgene expression cassette by *Chlamydomonas* regulatory elements. Several articles have reported that when the first intron of a gene was inserted in the 5'-upstream regions of that gene, a several-fold enhancement of gene expression was observed (Hirochika et al.). Therefore, to overcome this problem, we fused the full LHCSR3 gene, including exons and introns, into the luciferase gene and transformed this expression construct, as described in methods into *Chlamydomonas* C137 wild type cells. In this set of experiments, I successfully isolated a clone which exhibited stable and robust luciferase activity by incubating the cells under high light conditions. Southern blot analysis revealed there was a single copy insertion of expression cassette in the genome of reporter strain. I demonstrated that the bioluminescence activity of the reporter strain correlated well with the qE, endogenous *LHCSR3* gene expression, as well as the LHCSR3 protein accumulation pattern. Moreover, LHCSR3.1 showed rhythmicity under low light condition which implies LHCSR3-LUC reporter bioluminescence system is such sensitive that we could determine its rhythmicity under dim blue light, but red light did not show this rhythmicity possibly due to inhibitory effect of red light on LHCSR3 mRNA abundance as described above. I named the candidate clone $A4^+$ and used it as a wild-type strain in this study. To generate insertional mutants, the $A4^+$ strain was transformed with a DNA fragment containing a hygromycin-resistant gene (Berthold et al., 2002). We screened ~7,000 transformants which could grow on hygromycin plates and subjected them under strong blue light stress for 5 hours at 100 μmol m-2 s-1 followed by bioluminescence and qE analysis. Next, all the mutants were backcrossed to the $A4^-$ strain, and progenies were subjected to bioluminescence measurements and hygromycin resistance testing (cosegregation analysis). If the mutation was caused by a single integration of the marker gene into the genome, the mutant phenotype should have strictly cosegregated with the hygromycin resistance. 15 mutants have been isolated, identified and will be useful for future students.

Discussion: Our results support the hypothesis that red light is an important component of the visible spectrum involved in the circadian clock signalling network in *C. reinhardtii* by controlling changes in qE induction and LHCSR3 expression. If we clarify the impact of red light in natural environments, it may elucidate its important role and further reveal the importance of the circadian clock as a controller of photoprotection. Basically, in morning and evening time, red light is the major component of visible light. These changes throughout the day, where towards noon time the ratio of blue light dramatically increases. It has been pointed out that photodamage to the oxygen evolving complex of photosystem II (Ohnishi et al., 2005; Zavafer et al., 2015) tend to occur by irradiation with strong blue light intensity therefore the accumulation of LHCSR3 for dissipation of excessive energy is necessary during high blue light time periods. In the morning time the presence of LHCSR3 is not necessary since damage caused by blue light is less of a concern, so an unknown red light photoreceptor activates ROC75 to attenuate LHCSR3 expression. While as noon approaches, along with the damage potential of higher energy light, the increased blue light maintains degradation of the photoreceptor pCRY protein, which in turn results in attenuation of *ROC75* expression to permit activation of LHCSR3 expression. Working on this project, including the laboratory techniques I used, was a good foundation for my second project on PHOT dependent calcium signaling, as well as my third project designing and developing a robust and reliable high throughput screening method to identify mutants using forward genetics as fully described in chapter 3. My findings not only imply that cytosolic Ca^{2+} is the key factor for the integrated signal between PHOT and ETR pathways but also sheds more light on these pathways involvement in the regulation of LHCSR3 and photoprotection. My results show basal level of cytosolic Ca^{2+} treated with 1-hour illumination with blue light was elevated only in wild type cells but not the *phot* mutant. Thus, we can suggest that blue light-dependent Ca^{2+} signaling is dependent on PHOT. To elucidate further the mechanism behind regulation of *LHCSR3* gene expression, a high throughput forward genetic approach was used to screen mutants using bioluminescence derived from a luciferase reporter gene under intense blue light illumination. I could isolate 15 mutants that exhibit defect in calcium signaling pathway(s), blue-light-dependent signaling and photosynthetic electron flow. Our results present a broader image about the interplay between different signaling pathways to regulate *LHCSR3* gene expression.