# Redox and Light Derived Mechanisms of Environmental Perception are Utilized for Regulation of the Cyanobacterial RNA Helicase *crhR*

by

Sean Ritter

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Biological Sciences
University of Alberta

## Abstract

Cyanobacteria occur worldwide in environments that contain water and sunlight. They are crucial, positive players in carbon, oxygen and nitrogen cycling and function as producers of biosynthetic products such as biofuels. To optimize these processes, understanding mechanisms by which cyanobacteria sense environmental signals and regulate cellular processes is essential. Cyanobacteria obtain all of their energy from photosynthetic light harvesting. As such, the ability to genetically sense and respond to changes in their environment is essential for survival.

In bacteria, environmental sensing normally proceeds through stress-specific two-component signaling pathways. Despite the fact that the model cyanobacterial species, *Synechocystis,* encodes numerous stress specific two-component systems, a number of stress responsive genes are not regulated by any of these identified systems. Here, regulation of one of these orphan genes, the DEAD-box RNA helicase *crhR,* is described. Extensive evidence for an unexpected mechanism, by which divergent abiotic stresses are all sensed by their common effect on the redox potential of the photosynthetic electron transport chain, is provided. The convergent sensing mechanism fits the lifestyle of *Synechocystis*, allowing rapid and concerted changes in gene expression in response to a diverse abiotic stress. In addition, expression of *crhR* is demonstrated to be responsive to multiple distinct light regulated mechanisms at the transcriptional, translational and proteolytic levels, further supporting a crucial role for *crhR* in regulation of photosynthesis.

Finally, the specific site of the electron transport chain and the downstream signal transduction components which link changes in redox poise with altered gene expression

were investigated. The redox status of $Q_B$, a fixed stable quinone within photosystem II, and not freely diffusible plastoquinone, is the specific site of physiological change which facilitates redox regulation of *crhR.* Evidence for involvement of the *hik8/rpaA/rpaB* two-component signaling system in regulation of *crhR* is also provided. Hik8 has been demonstrated to function downstream of the KaiABC circadian oscillator complex in *Synechocystis,* suggesting implications of the circadian clock in regulation of *crhR* expression. On an evolutionary scale, evidence for related systems in *Staphylococcus aureus* and plant chloroplasts suggests that convergent sensing mechanisms may occur more widely than anticipated.

## Preface

Chapter 2 of this thesis has been published as Ritter et al., "Evidence for convergent sensing of multiple abiotic stresses in cyanobacteria" *BBA- General Subjects,* Vol. 1864, issue 1, 2019. Western analysis was performed by S.P.A. Ritter, A.C. Lewis, S.L. Vincent, L.L. Lo, A.P.A. Cunha and D. Chamot. All data displayed aside from Figure 2 was generated by S.P.A Ritter. PAM fluorometry experiments were performed by S.P.A. Ritter, I. Ensminger, G.S. Espie and G.W. Owttrim. The manuscript was written by S.P.A. Ritter, A. Lewis, D. Chamot, G.S. Espie and G.W. Owttrim.

Chapter 3 of this thesis has been submitted for publication. Western analysis was performed by S.P.A Ritter, S.L. Vincent, A.R.R Rosana, and A.C. Lewis. qPCR was performed by S.P.A. Ritter. Northern analysis was performed by A.R.R. Rosana. All data displayed aside from Figures 4 and 8 was generated by S.P.A. Ritter. Experiments were designed by S.P.A. Ritter, A.R.R. Rosana and G.W. Owttrim. The manuscript was written by S.P.A. Ritter and G.W. Owttrim.

Chapters 1, 4, 5, and 6 of this work are original work by S.P.A. Ritter.

**Acknowledgements**

This thesis would not be possible if not for the help of many people, some of which I would like to thank directly:

Dr. George Owttrim, for providing me the opportunity and freedom to answer research questions I am passionate about. It is difficult to imagine my graduate experience without your camaraderie, guidance and unwavering belief in my ability as researcher. I will sincerely miss having a supervisor as supportive as you are, both inside the lab and out.

My fellow members of the Owttrim Lab. To Denise Whitford, thank you for teaching me the fundamentals of molecular biology and instilling an atmosphere of support in the lab. To Brendan Whitman, thank you for your commiseration and guidance on what to do when things go wrong. And to Logan Brand, thanks for always being willing to chat, whether it be about science or life. Sitting on top of Mount Royal post-Plant Biology and down on the beach post-ISPP and are fond memories I won't soon forget.

All those who guided me through the technical aspects of research, especially Troy Locke for your always positive attitude and qPCR wizardry which made developing a protocol as close to a painless process as I could imagine, and Dr. George Espie for your expertise, patience and enthusiasm about cyanobacterial physiology which made my time spent in your lab one of the scientific highlights of my degree.

The members of my supervisory committee, Dr. Glen Uhrig and Dr. Tracy Raivio for your time, input, and advice on both my thesis and future aspirations.

And to my family: My Sister, for always be there for me and inspiring me to persevere through challenging situations. My Father, for unconditionally supporting me and trusting my judgment, despite sometimes knowing better. Finally, to my Mother, for your seemingly bottomless capacity for understanding, care and support. Everything I achieve is because of you.

## Table of Contents

## List of Figures

## List of Tables

# Chapter 1: Introduction

## 1.1 Cyanobacteria as a model photosynthetic organism

Cyanobacteria comprise a diverse group of free living Gram-negative oxygenic photoautotrophs which are key contributors to primary productivity worldwide (Plohnke et al., 2015). An incredibly diverse phylum, various cyanobacteria are found anywhere water and light are available, and are capable of a combination of oxygenic photosynthesis, nitrogen fixation and terminal differentiation (Kopf and Hess, 2015). This diversity is reflected at the genomic level as well; genome sizes vary from 1.44 Mbp in Candidatus *Atelocyanobacterium thalassa* (formerly UCYN-A) to 21 Mbp in *Scytonema hofmanni* PCC 7110 (Dagan et al., 2013; Bombar et al., 2014). Due to the wide range of genetic diversity of cyanobacteria, a number of strains have been utilized as model systems. For example, due to pronounced changes in response to varying light conditions, *Synechococcus* sp. strain PCC 7942 has been utilized extensively for analysis of the circadian clock (Iwasaki et al., 2000; Kim et al., 2012; Espinosa et al., 2015). The multicellular strains *Anabaena* sp. strain PCC 7120 and *Fremyella diplosiphon* UTEX 481 are used to study nitrogen fixation and complementary chromatic adaptation (CCA) respectively (Rosinski et al., 1981; Grossman, 2003; Bussell et al., 2013; Nasser et al., 2015; Wiltbank et al., 2018). Natural competence towards transformation, a sequenced genome (the first available photosynthetic organism), reasonable growth conditions, genetic tractability, and the ability to switch between photoautotrophic and mixotrophic growth in the presence of glucose make the single celled cyanobacteria *Synechocystis* sp. strain PCC 6803 (*Synechocystis)* a useful model organism for the study of photosynthesis and biotechnological applications (Kaneko et al., 1996).

As a photoautotroph, when light is available *Synechocystis* can be grown on defined minimal media (typically BG-11) devoid of any fixed carbon source (Owttrim, 2012). As an obligate photoautotroph, light harvesting combined with oxygenic photosynthesis is utilized to generate all the energy and carbon skeletons required for growth (Lau et al., 2015). This conversion of light energy into chemical energy generates ATP and NADPH via the conversion of water to oxygen and is catalyzed by biochemically defined membrane complexes which span the thylakoid membrane (Nelson and Ben-Shem, 2004). These make up the electron transport chain (ETC), the central component of Synechocystis' metabolism. Light harvesting occurs within two photosystems, photosystem II (PSII),

which provides the majority of excited electrons during normal growth conditions and Photosystem I, which is better suited towards low light conditions (Chitnis et al., 1993). A schematic of the cyanobacterial ETC is provided as Figure 1.1

PSII is the primary light harvesting complex of the ETC. A variety of light harvesting pigments are sequentially excited via light, eventually converging on the reactive center of chlorophyll *a*, P680 (Govindjee and Shevela, 2011). In cyanobacteria, most light harvesting is performed by the phycobilisomes (PBS) (Grossman *et al.,* 1993). Phycobilisomes consist of stacked rods of the pigment phycocyanin (PC) with an allophycocyanin core that maximally absorb green-orange light (MacColl, 1998). Phycobilisomes primarily associate with PSII but also excite the P700 chlorophyll complex in PSI (Mullineux, 1992). Transient disassociation of phycobilisomes from either PSI or PSII, termed state transition, facilitates allocation of light energy throughout the ETC (Joshua and Mullineaux, 2004). This process of photoadaptation is complemented by the photoactive orange carotenoid protein, which in its active form can bind the PBS and absorb excess excitation energy, reducing photochemical damage (Kerfeld and Kirilovsky, 2011).

PSII consists of 17 intrinsic and three extrinsic subunits (Guskov et al., 2009). Most importantly, two ~40-kDa proteins, D1 (*psbA)* and D2 (*psbB)* form the PSII reaction center and facilitate electron transport from chlorophyll to plastoquinone (PQ) (Nixon et al., 2010). Using energy obtained from light harvesting, PSII oxidizes two water molecules to generate molecular oxygen and four protons. This is aided by the three extrinsic subunits of PSII (PsbO, PsbU and PsbV), which all contribute to the stability of the $CaMn_4$ water splitting complex to facilitate generation of high energy electrons (Enami et al., 2008). Two electrons and two protons are transferred onto the primary stable quinone ($Q_A$), then to the secondary quinone ($Q_B$) and eventually to the reduced cofactor PQ. Relative amounts of oxidized plastoquinone (PQ) and reduced plastoquinone ($PQH_2$) determine the redox potential of the PQ pool. $PQH_2$ is lipid diffusible facilitating the transfer of electrons from PSII to cytochrome-$b_6f$ (cyt-$b_6f$).

The most striking feature of cyt-$b_6f$ is the similarity to that of its mitochondrial relative, highlighting the similarities between photosynthesis and respiration (Hurt and Hauska, 1981). $PQH_2$ is oxidized at the $Q_O$ site of cyt-$b_6f$ which facilitates the pumping of

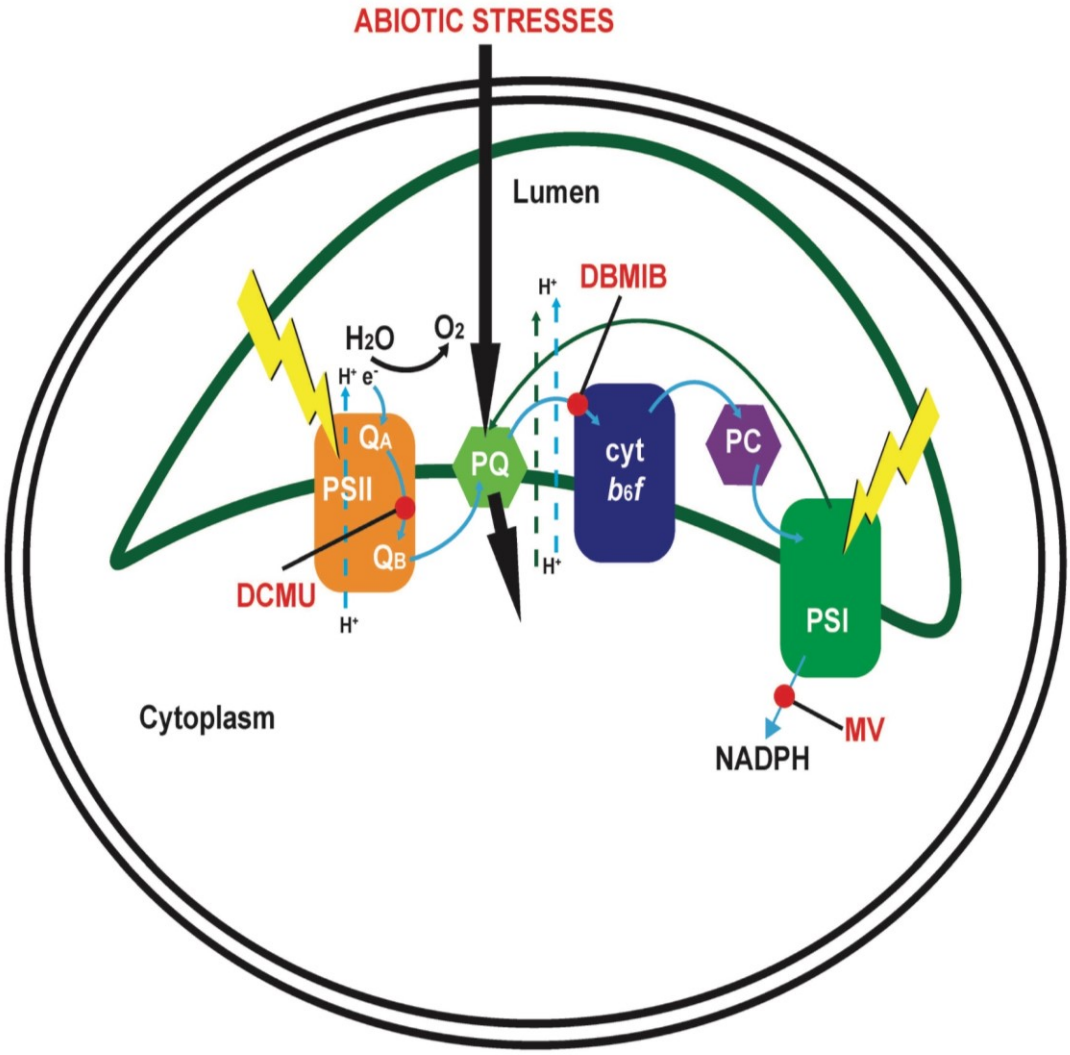



**Fig. 1.1. Overview of the cyanobacterial photosynthetic electron transport chain**. Figure adapted from Ritter et al., 2019. Light energy initially absorbed by phycobilisomes and PSII is used to transfer electrons, ultimately derived from the water-splitting complex, to an electron transport chain. The electrons flow in a linear pathway (blue arrows) from PSII through PQ to cytochrome b6f into PC and then to PSI. PSI utilizes light energy to transport electrons across the thylakoid mem- brane to reduce ferredoxin and ultimately NADP+ used for carbon fixation. Linear electron flow also pumps H+ ions into the lumen that are used for the chemiosmotic production of ATP. Electrons can also enter the chain via a cyclic electron transport pathway from PSI to PQ thereby enhancing ATP production at the expense of NADPH (green arrow). PSII, photosystem II; PC, plastocyanin; cyt b6f, cytochrome b6f complex; PQ, plastoquinone; PSI, photosystem I. Inhibitors that alter electron transport through the chain are indicated in red. DCMU blocks electron transfer from QA to QB in PSII, DBMIB prevents reduction of cytochrome b6f from PQ and MV prevents electron transfer from PSI to NADP+ . Figure originally adapted from Berla et al., (2015).

protons across the thylakoid membrane. The resulting proton motive force is later utilized by ATP synthase to generate ATP (Nelson and Ben-Shem, 2004). Electrons are passed from cyt-$b_6f$ to plastocyanin, which serves as a branching point for electrons between linear electron flow, primarily through cytochrome C oxidase to generate $H_2O$ during respiration, and re-excitation at PSI for cyclic electron flow during photosynthetic carbon fixation (Howitt and Vermaas, 1998). In *Synechocystis*, two alternative oxidases (quinol oxidase and alternative respiratory terminal oxidase) have also been described as accepting electrons during rapidly changing light conditions, providing an example of how electron flow can vary depending on environmental conditions (Lea-Smith et al., 2013).

Unlike higher plants, components of the photosynthetic and respiratory electron transfer apparatus are shared in cyanobacteria, with respiratory electrons entering at the PQ pool (Hirano et al., 1990). Respiratory electron flow was proven to be capable of rescuing state transition, the process by which energy is allocated between the two photosystems, in *Synechococcus* sp. PCC 6301, one of the first descriptions of a process responsive to the redox poise of the ETC (Mullineaux and Allen, 1986). Similarly, under conditions where PQ is oxidized, such as darkness, expression of the NADPH dehydrogenase supercomplex increased, thereby shuttling electrons from the ETC to water during respiration (Ma et al., 2007). This complex facilitates cyclic electron flow though PSI and as a result aids in maintaining homeostasis of the redox poise of the PQ pool, which has been suggested to be tightly controlled (Schuurmans et al., 2014). Thus, changes in the redox poise of PQ have been demonstrated to enable changes in gene expression. Similar mechanisms have been described in pea chloroplasts, suggesting that PQ mediated redox regulation has been maintained since the original endosymbiotic event which lead to plant chloroplasts (Tullberg et al., 2000).

PSI reduction status is affected both by receiving electrons from either cyt-$b_6f$ or by generation of electrons through light harvesting. PSI has the ability to modulate its antennae of light harvesting pigments, allowing both increased flexibility in which light can be used, and optimized light harvesting under varying light intensities (Melkozernov et al., 2005). Ferredoxin (Fd) accepts electrons from PSI. Fd can either donate electrons to ferredoxin-NADP reductase (FNR) which generates NADPH or back into cyt-$b_6f$ via cyclic electron transfer, generating ATP via proton motive force. As a result, PSI generates both

ATP and NADPH, while PSII produces only ATP (Howitt et al., 2001). This is essential for photosynthetic organisms in order to balance the ratio of ATP/NADPH required for carbon fixation via the Calvin-Benson cycle. Indeed, the requirement for both ATP and NADPH for carbon fixation has been interpreted to suggest that PSI evolved before PSII (Baymann et al., 2001). This evolutionary model is supported by the high degree of conservation in PSI components between cyanobacteria and higher plants. The redox poise of the Fd pool functions similarly to the PQ pool and has been observed to regulate gene expression (Ceccoli et al., 2012). To what extent these two cofactor pools regulate gene expression remains understudied.

## 1.2 Measurement of ETC activity in cyanobacteria

Characterization of the cyanobacterial ETC has relied heavily on pulse amplitude modulated (PAM) fluorometry. Modulated fluorometers specifically detect and amplify chlorophyll fluorescence excited by a specific wavelength of light (Campbell et al., 1998). Initially, a weak constant measuring light which is sufficient to drive photosynthesis is applied in order to determine basal levels of fluorescence in dark-adapted cells. By comparing changes in fluorescence yield in response to changes in the intensity of actinic light (which drives photosynthesis) and intermittent saturating light pulses (which close all PSII centers) ratios representative of photosynthetic activity can be derived.

Classically, cyanobacterial PAM studies have utilized measurements of photosynthetic activity previously verified in higher plants. However, a recent study by Ogawa et al. (2017) demonstrated that fundamental differences arising from respiratory electron flow and varying pigment composition can lead to inaccurate comparisons between cyanobacterial species and strains. For instance, in the dark, higher plants cease to perform photosynthesis, and PQ subsequently becomes completely oxidized. This is not the case in cyanobacteria, as in the dark the oxidization state of PQ remains relatively reduced due to the contribution of respiratory electron flow. To overcome this difference in the dark-adapted PQ redox poise between plant and cyanobacteria, an additional step is required to accurately utilize PAM with cyanobacteria. Cells are illuminated with blue light, which should preferentially excite PSI and therefore relatively oxidize the PQ pool (Schreiber et al., 1995).

Similar issues also arise when determining other photosynthetic parameters using PAM. In higher plants, the presence of actinic light causes a rapid increase that generates maximum levels of fluorescence (Fm) in the light, which subsequently dissipates via a process termed non-photochemical quenching (NPQ) (Ogawa et al., 2017). However, in cyanobacteria a small, persistent increase in fluorescence is observed This suggests a difference between light acclimation systems in land plants and cyanobacteria, where NPQ is not immediately induced for energy dissipation (Misumi et al., 2016). Incubation with the ETC inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks electron transfer from $Q_A$ to $Q_B$ within PSII and therefore leads to complete oxidation of the PQ pool, is subsequently necessary to determine the "true" $F_m$ in cyanobacteria (Campbell et al., 1998). Incubation with the ETC inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks electron transfer from $Q_A$ to $Q_B$ within PSII and therefore leads to complete oxidation of the PQ pool, is subsequently necessary to determine $F_m$ in cyanobacteria (Campbell et al., 1998). DCMU has been shown to decrease oxygen evolution and qP, a measure of relative flow of electrons in and out of PSII, in a concentration dependent fashion, thus making it a potent inhibitor of photosynthesis (Campbell et al., 1998). A second inhibitor, dibromothymoquinone (DBMIB), inhibits oxidation of $PQH_2$ at the $Q_O$ binding site within cyt-*b6f* (Preston and Critchley, 1998; Roberts and Kramer, 2001). Thus, DBMIB causes a net reductive effect on the ETC upstream of $Q_O$, including $Q_B$ and PQ. The opposing effects of DCMU and DBMIB, specifically oxidation and reduction of the PQ pool respectively, have frequently been interpreted to indicate that the redox status of PQ is a major regulator of photosynthetic physiology. For example, these ETC inhibitors were utilized to verify the redox regulation of state transitions. Whole cell absorbance measurements confirmed that illumination with wavelengths of light favoring PSI, the expected increase in PSI absorbance is impeded in DBMIB treated cells, but not in the presence of DCMU (Allen and Mullineux, 1990). This confirms redox regulation of state transition, based on the differential effect of these two inhibitors on the PQ pool redox poise.

Despite having similar effects on qP, DBMIB has also been shown to cause a concentration dependent rise in NPQ, which does not increase in the presence of DCMU (Campbell et al., 1998). Non-photochemical quenching (NPQ) is a measure of all excited

electrons which do not drive oxygenic photosynthesis but are also not lost as heat. In cyanobacteria, NPQ is primarily achieved through the previously described process of state transition, as well as induction of other photoprotective processes such as direct transfer of electrons from the light harvesting phycobilisomes onto orange carotenoid protein (Boulay et al., 2010). Finally, all electrons not utilized though photochemical and non-photochemical processes are presumed to be lost as heat in cyanobacteria (Campbell et al., 1998). This is represented by the parameter Φf, d. By comparing qP, ΦNPQ and Φf, d values, an overall representation of electron allocation in cyanobacteria can be obtained.

## 1.3 Abiotic stress signal transduction pathways – two component signaling mechanisms

For free-living organisms in the environment, integrating, processing and responding to multiple abiotic stress signals that they continuously encounter is essential for survival. Reacting to varying environmental conditions requires both specific responses where physiology is altered to address the specific stress present, as well as a more general metabolic response which is shared between stresses. Bacteria typically utilize two-component signaling systems to sense and enact genetic changes to specific changes in the environment (Beier and Gross, 2006; Capra and Laub, 2012). In response to an environmental stimulus, a histidine kinase (HK) auto-phosphorylates, leading to activation of either one or two cognate response regulators (RRs), depending on the kinase. The *Synechocystis* genome encodes 47 and 42 HKs and RRs, respectively (Ashby and Houmard, 2006). This HK-RR repertoire, expanded relative to higher plants, is considered to be a reflection of the diversity of environments inhabited by cyanobacteria. Since two-component systems are generally stress specific, bacteria tend to encode two-component signal transduction pathways in a similar number to abiotic variables encountered (Jung et al, 2012).

Of the 47 HKs encoded by *Synechocystis,* microarray data has suggested that five are responsible for the perception of salt stress in *Synechocystis.* Hik33, Hik10, Hik34, Hik16 and Hik2 were all shown to regulate unique sets of genes responsible for the hyperosmotic stress response (Marin et al., 2003; Paithoonrangsarid et al., 2004). However, salt inducibility of ~ 80% of tested genes previously shown to be salt responsive was

unaffected in the absence of any of the tested HKs. This suggests that genes which facilitate response to diverse abiotic stresses can respond to common, indirect effects on cell physiology such as reduction of the redox poise around PQ. Similarly, only 38 of the 109 tested cold-shock responsive genes are regulated by the low temperature sensor Hik33 (Sinetova and Los, 2016). Importantly, these observations demonstrated that a significant number of stress inducible genes are not regulated by the traditional stress specific two-component signaling systems in *Synechocystis* and thus additional unidentified sensing mechanisms are involved.

Hik33 is a well-characterized example of an HK involved in regulation of divergent stress responsive genes via two RRs. Stress sensing by Hik33 involves signaling through Rre26 to facilitate changes in cold responsive gene expression, while signaling through a second response regulator, Rre31, enacts changes in expression of a different regulon of osmotic and salt responsive genes (Suzuki et al., 2000; Mikami et al., 2002; Shoumskaya et al., 2005). Signaling associated with alterations in membrane fluidity caused by both temperature and osmotic stress also induce Hik33 (Maksimov et al., 2017). In addition, Hik33 also plays a role in the oxidative stress response through interactions with the transcription factor PedR (Kanesaki et al., 2007). Thus, Hik33 is an example of a cyanobacterial HK that is activated by diverse stresses leading to regulation of divergent regulons via signaling through two RRs.

An additional HK with a previously characterized role in response to diverse abiotic stresses is Hik2. Hik2 is an essential kinase in cyanobacteria and represents one of only four Hiks (along with Hik33, Hik8 and Hik34) which are conserved among all cyanobacteria (Kobayashi et al., 2017). Hik2 was originally characterized as playing a role in response to osmotic stress (Paithoonrangsarid et al., 2004). In order to circumvent the essential nature of Hik2, Kotajima, Shiraiwa and Suzuki (2014) generated a fusion protein with the signal input domain of Hik2 linked with the kinase output domain of the unrelated Hik7. This hybrid kinase was shown to be activated by a wide range of salts, confirming the earlier microarray data that Hik2 was involved in the osmotic response. Subsequently, *in vitro* assays have shown that Hik2 is capable of binding quinones, indicating that the ETC redox poise may play a role in kinase activation via salt stress (Ibrahim, et al., 2016). Hik2 also exhibits significant homology to the chloroplast stress kinase CSK, an ancestral

kinase found in higher plants thought to arise from the original endosymbiotic cyanobacterium (Puthiyaveetil et al., 2008). Like Hik2 in cyanobacteria, CSK is required in higher plants for gene regulation leading to photosystem stoichiometry adjustment in response to changes in the redox poise of the PQ pool, potentially coupling gene expression with photosynthesis.

## 1.4 Redox signaling systems as convergent sensing mechanisms

Treatment of *Synechocystis* with the previously described photosynthetic inhibitors DCMU and DBMIB, which differentially affect the redox poise around PQ/$Q_{B,}$ has been shown to alter the expression of genes involved in a variety of processes, including the heat shock proteins *groEL, dnaK, clpB,* and *hspA* (Hihara et al., 2003). As the redox poise of the ETC has been shown to be affected by diverse abiotic stresses, this offers a way for cells to integrate multiple environmental signals into a single point of perception, as described in Ritter et al. (2019). This **convergent sensing** model of abiotic stress perception contrasts the standard model of stress response, which utilizes multiple stress specific signaling pathways functioning in parallel, eventually leading to redundant expression of genes required for both abiotic stresses, representing a **convergent signaling** mechanism (Fig 1.2).

Multiple redox responsive signaling systems have been shown to independently facilitate adaptation of a diverse range of photosynthetic processes in response to changing environmental conditions in *Synechocystis*. In 1991, Mohamed and Jansson showed that levels of the *psbA* transcript, which encodes for the key D1 peptide within photosystem II, was induced by high light and stabilized by darkness. *psbA* was later shown to have an extended half-life in the presence of DBMIB, suggesting a light independent, redox mediated signal may be controlling degradation (Alfonso et al., 2000). Regulation of *psbA* via the redox poise of the PQ pool was further supported through genetic analysis of a *rpaA* deficient strain. RpaA is the primary output of the circadian clock in cyanobacteria and is associated with adjustment of photosystem stoichiometry (Li and Sherman, 2000). *psbA* displayed constitutive high expression in *rpaA* mutant strains, suggesting release from repression (Hsiao et al., 2004) Similarly, mutations in genes crucial for efficient photosynthesis such as phycobilisomes (El Bissati and Kirilovsky, 2001) have also been

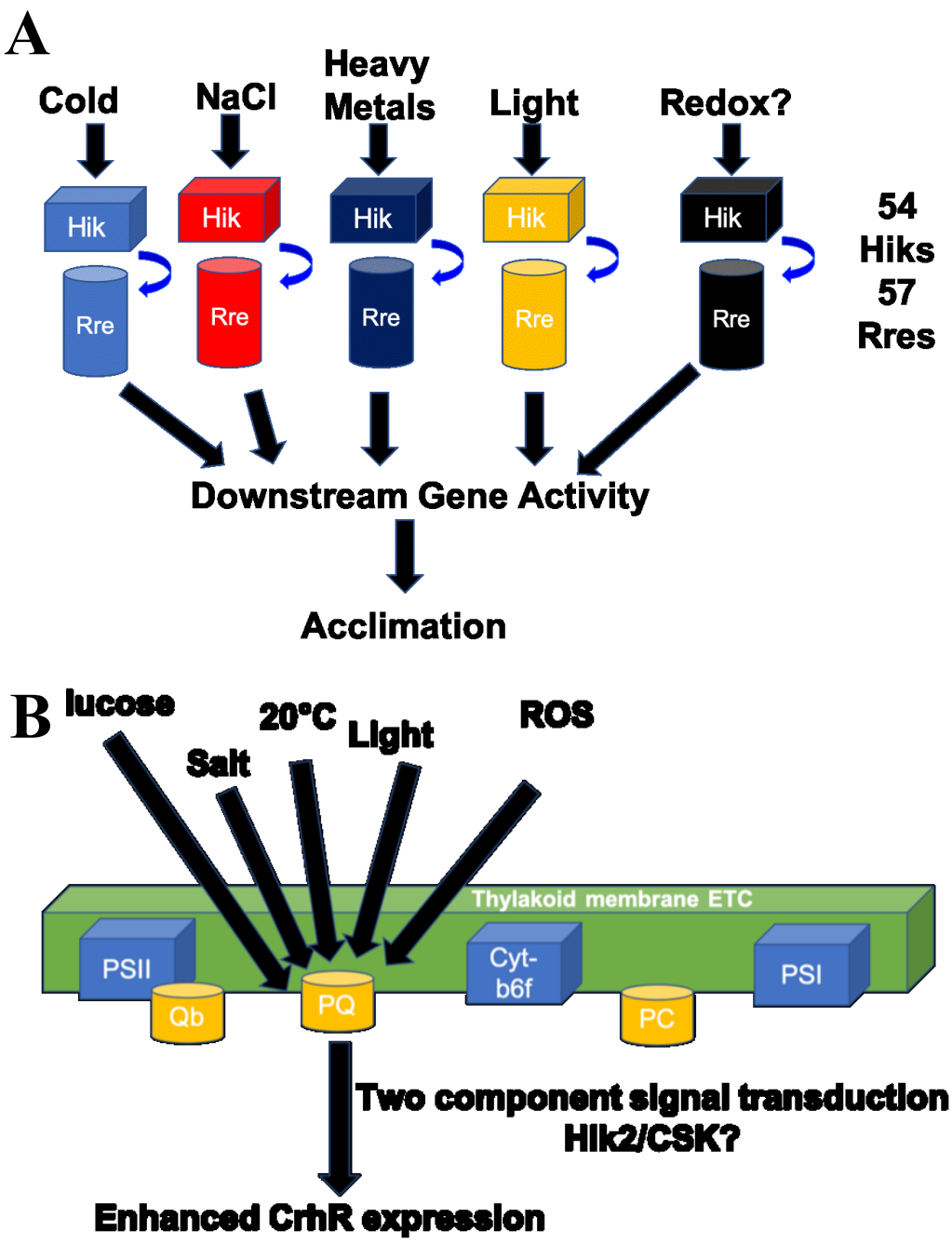



**Fig. 1.2. Overview of convergent signaling and convergent sensing mechanisms.** (A) Convergent signaling, where multiple separate histidine kinases – response regulator pairs respond to individual abiotic stresses. Response to multiple stresses is integrated at the promoter level. (B) Convergent sensing, where a single histidine kinase is responsive to changes in the redox poise of the electron transport chain caused by diverse abiotic stress. Response to multiple stresses is integrated upstream of the promoter at the level of physiology.

shown to affect the expression of *psbA*, further linking gene regulation with the redox potential of the ETC.

As PAM fluorometry is only able to detect excitation from $Q_A$ in PSII, individual differences in the redox poise of specific electron carriers within the ETC that may facilitate redox signaling remains undetermined. A variety of processes, such as state transition have been described as PQ regulated despite failing to differentiate between the redox poise of $Q_A$ and $Q_B$ (Fufezan et al., 2007). To circumvent this problem, exogenous quinones with varying redox properties can be employed. While the majority of tested quinones are capable of accepting electrons from around PSII, not all quinones are capable of accepting electrons directly from $Q_B$ (Fu *et al.,* 2017). This is most likely a result of incorrect positioning within the $Q_B$ binding pocket, as changing PSII structure has been shown to both increase and decrease electron transfer (Ermakova-Gerdes and Vermaas, 1998; Guskov *et al.,* 2009; Luo *et al.,* 2014; Fu *et al.,* 2017). Only one report has suggested that changes in the redox poise of qB is the signal required to initiate D1 repair, as this did not occur in the Δ*psbH* strain of 6803 which suffered increased $Q_B$ photodamage (Komenda and Barber, 1995). Similarly, the $Q_C$ protein which encodes for the hydrophobic quinone binding tunnel within PSII, causes defects in state transitions when ablated, suggesting a potential role in gene regulation alongside $Q_B$ (Huang et al., 2016).

Light perception mechanisms which are not associated with ETC redox potential also have a profound effect on gene expression in *Synechocystis*. An example is expression of the co-transcribed operon of *cph1/rcp1,* which does not exhibit sensitivity to DCMU and DBMIB but is significantly upregulated in the dark (Garcia-Dominguez et al., 1999). This suggested regulation is directly light responsive and proceeds independently of the ETC. *cph1/rcp1* has also been shown to facilitate diverse metabolic change in response to the red to far-red light shift (Hubschmann et al., 2005). Another example of a requirement for a light signal to initiate regulation of gene expression is associated with D1 translation. In *Synechocystis*, the cytosolic *psbA* transcript is bound in a pre-initiated state by ribosomes in the dark. A light signal is then required to target the pre-initiated ribosomes to the thylakoid membrane where elongation of full length D1 protein occurs (Aro et al., 2001). This distinction illustrates the fact that *Synechocystis* perceives its environment through

signaling mechanisms responsive to both light directly and light-driven redox regulation, imparting regulation at both the transcriptional and post-transcriptional levels.

Similar regulatory networks function within higher plant chloroplasts to adjust photosynthesis to changing environmental conditions. Translation of the chloroplast *psbA* mRNA is controlled by divergent signals; one from PSII which is dependent on the redox status of the PQ pool, and one which derives from PSI and is mediated by a redox active ferredoxin (Trebitsh and Danon, 2000). This requires ferredoxin to be sensed locally, as redox poise is directly linked to conditions within the thylakoid membrane. The CoRR hypothesis (Co-location for Redox Regulation) suggests that local response to ETC redox changes is crucial for maintenance of photosynthetic efficiency. This is proposed to be the reason for retention of a limited genome in chloroplasts, as this allows for direct and rapid coupling between redox state and gene expression (Allen, 2017).

## 1.5 CrhR: A D-E-A-D Box RNA helicase

RNA helicases have been identified in all kingdoms of life and are highly conserved motor proteins that utilize ATP hydrolysis to rearrange secondary structure of RNA transcripts and ribonucleoprotein complexes (Linder et al., 2011; Owttrim, 2013). RNA helicases use ATP to bind to and then unwind mostly short (>12 bp) RNA duplexes using an unknown mechanism (Jarmoskaite and Russell, 2014). Binding to RNA structure confers functionality through a clamp type action where the helicase functions as a scaffold, facilitating the recruitment of additional modifying proteins (Linder and Jankowsky, 2008). Recruited proteins can then enact further changes in structure, significantly affecting the activity of the RNA. (Ballut et al., 2005). These activities allow for RNA helicases to function in diverse aspects of RNA metabolism, commonly affecting transcription, translation initiation, ribosome assembly, mRNA splicing and RNA maturation and degradation in all organisms (Rocak and Linder, 2004). It has been proposed that in processes such as prokaryotic ribosome biosynthesis, a single RNA helicase will be associated with a transcript for its entire lifespan, from transcription to degradation (Martin et al., 2013).

Helicases are grouped into two superfamilies, with all RNA helicases within superfamily 2 (SF2) (Linder and Jankowsky, 2011). The largest of the five subfamilies

which make up SF2 are the DEAD-box RNA helicases which contain the characteristic Asp-Glu-Ala-Asp DEAD-motif within a conserved helicase core consisting of two RecA-like domains and ~12 conserved short amino acid domains (Fairman-Williams et al., 2010). The number of DEAD-box RNA helicase genes encoded in bacterial genomes varies greatly, from zero in *Mycobacteria* to ten in the Gamma-proteobacteria *Shewanella paeleana* ATCC 700345 (Linder, 2014). The Synechocystis genome encodes a single DEAD-box RNA helicase, *crhR*, suggesting that the range of functions performed by CrhR may be expanded (Owttrim, 2013).

CrhR has been observed to facilitate multiple processes, including photosynthesis at low temperatures, reducing the expression of heat shock proteins in response to cold stress, and autoregulation of its own translation and proteolysis (Rosana et al., 2012a; Rosana et al., 2012b). These changes are enacted by a low processivity RNA unwinding activity (RNA substrates < 41 bp) that bidirectionally unwind duplex RNA (Chamot et al., 2005). Different from the majority of RNA helicases, CrhR can also anneal RNA duplexes which, when combined with unwinding activity, catalyzes RNA strand exchange (Chamot et al., 2005).

Truncation of *crhR* (*crhR*$_{TR}$) causes cessation of growth and decreased viability of *Synechocystis* under cold stress (Rosana et al., 2012a). At 20ºC, photosynthetic carbon fixation halted in *crhR*$_{TR}$, despite normal uptake of inorganic carbon. Oxygen exchange rates and photosynthetic pigment composition both decreased in *crhR*$_{TR}$ regardless of temperature, suggesting decreased overall photosynthetic capacity. Deletion of *crhR* was also associated with impaired state transitions due to down regulation of PSI reaction center genes *psbB* and *psbL* (Sireesha et al., 2012). Additional genes involved in photosynthesis and energy metabolism, such as *flv3/4,* which is involved in photoprotection around PSI/PSII respectively, and the ncRNA PmgR1 which is required for the switch to heterotrophic growth are also misregulated in the *crhR* mutant background (Georg et al., 2019). Similarly, low temperature inducible expression of the heat shock protein chaperone encoding genes *groEL1* and *groEL2* was reduced in the *crhR* mutant, suggesting a role in the general stress response (Prakash et al., 2010). In conjunction, proteomic data suggests that deletion of *crhR* causes a stress phenotype unrelated to cold, as proteins such as Sll1621 (glutathione-dependent peroxidase) were found to be

overexpressed in the mutant even at the normal growth temperature of 30ºC (Rowland et al., 2011). Indeed, differences in cellular morphology were also observed under normal growth conditions in the *crhR*$_{TR}$ strain (Rosana et al., 2012a). *crhR* has also been shown to be induced by NaCl in addition to cold stress, suggesting it may play a more expanded role in the adaptation of photosynthesis to diverse abiotic stress beyond the temperature response (Vinnemeier and Hagemann, 1999).

In 2000, Kujat and Owttrim observed that *crhR* expression decreased in the dark but could be partially rescued by supplementation with glucose. Provision of 3-OMG, a glucose analogue which is actively transported into the cell but not metabolized by *Synechocystis,* did not restore *crhR* expression in the dark. Thus, an aspect of glucose metabolism, specifically respiratory derived electrons that enter the ETC at PQ was responsible for rescue. Treatment with DCMU mimicked placing the cells in darkness, while treatment with DBMIB allowed for normal expression of CrhR. This differential expression in the presence of either ETC inhibitor at both the transcript and protein level suggested that *crhR* expression is responsive to the redox poise of the ETC around PQ. Through a proteomic approach where *Synechocystis* cells were subject to 22ºC cold stress for a period of 3 hours in both the light and dark, Chen et al (2018) identified a significant number of proteins for which light was essential for their cold responsiveness, including CrhR. This suggested that the interaction between abiotic stress signaling and redox conditions may be more complex and expanded than previously thought. The role of redox sensing and signal transduction in response to varying abiotic stress and light conditions on expression of the DEAD-box RNA helicase CrhR is the focus of this thesis.

## 1.6 Thesis objectives

The primary objective of this thesis is to further determine the relationship between CrhR expression and both redox and light mediated mechanisms of environmental perception in *Synechocystis*. This was performed by establishing a link between abiotic stresses and varying growth conditions known to induce CrhR and the ETC, describing a unique role for light quality and quantity in *crhR* expression, and analysis of quinone signaling and redox responsive two-component signaling mutants.

Previous work performed in the lab established *crhR* expression as being redox regulated, despite being classically described as a cold-shock gene. *crhR* had also been shown to be induced by NaCl, suggesting that diverse abiotic stresses may be capable of causing induction. Taken together, these two observations suggested that the redox poise of the ETC may be similarly affected by diverse abiotic stress, which can then enact changes in downstream gene regulation. The work presented here shows that regulation of CrhR is a product of convergent sensing, and not convergent signaling, of abiotic stress via the redox poise of the ETC. CrhR is shown to be induced at the protein level by divergent abiotic stressors including cold shock, osmotic stress and heavy metal stress. CrhR is not induced in response to oxidative stress, suggesting the mechanism of CrhR induction is not general to all abiotic stresses. Through the use of a variety of specific ETC inhibitors, this work shows that reduction of the area $Q_B$/PQ is required for CrhR induction in response to abiotic stress, while the redox potential around PSI/Fd is not involved. Finally, a link between altered photosynthetic activity and abiotic stress capable of inducing CrhR is established via PAM fluorometry. Diverse abiotic stresses are shown to be capable of enhancing reduction of the ETC, leading to conditions that induce CrhR.

Using an interlinked experimental setup defined by Barth et al., in 2014, we confirm that CrhR is redox regulated, but with an additional light signal required for its expression at the protein level. Stimulation of photosynthesis with increased light intensity further confirmed the link between the redox poise of the ETC and CrhR expression. An additional level of regulation in response to darkness is also described, with light being required for transcription, translation and proteolysis of CrhR. The role of light quality in the expression of CrhR at the levels of transcription, translation, and proteolysis of CrhR was also investigated. CrhR is not degraded, transcribed, or translated in response to cold shock when in the dark. Cells were subjected to either blue, red or white light either during temperature down or upshift. Through both western blot and qPCR analysis, it appears that while both transcription and proteolysis are simply light dependent, redox regulation is imparted at the level of translation as red but not blue light is sufficient for induction. *crhR* regulation at the level of translation introduces yet another level of gene regulation. A model for describing how CrhR may play a role in its own regulation is described. The redox regulation hypothesis is also confirmed by the contribution of respiratory electron

flow from glucose metabolism enhancing reduction of PQ in the dark being correlated with crhR expression at both the transcript and proteins levels.

Analysis of the signal transduction mechanism by which the previously identified convergent sensing mechanism contingent on the redox poise around PQ leads to induction of CrhR was also performed. Utilization of the exogenous quinones PPBQ, DMBQ and DCBQ, all of which accept electrons under slightly different conditions, suggests that the redox poise of $Q_B$ is specifically responsible for CrhR induction. Thorough characterization of redox-responsive two-component signaling systems was performed, further establishing a link between the ETC and the promoter of CrhR. Despite testing numerous two-component signaling systems previously linked to redox regulation, CrhR was stress inducible and degraded under expected conditions in all tested strains. However, changes in the kinetics of CrhR induction suggest the *hik33/rpaA/rpaB* signaling pathway may be involved.

Throughout this thesis, the specific physiological changes responsible for induction of the RNA helicase gene, *crhR*, are described. Changes in the redox poise of the ETC around $Q_B$/PQ caused by diverse abiotic stress and varying light conditions are capable of CrhR induction, as well as altered growth conditions such as high light. Light also plays a unique role in the induction of CrhR, proteolysis is strictly light dependent, while light quality specific control appears to be imparted at the level of translation. Redox control at the transcriptional level appears to arise from changes at $Q_B$ and may proceed through the *Hik33/RpaA/RpaB* two-component signaling pathway, suggesting involvement of *crhR* in regulation of circadian gene expression. Initially characterized as a cold shock helicase, CrhR clearly plays a more expanded role in maintaining cellular homeostasis in response to changing environmental conditions. CrhR RNA helicase activity has the potential to regulate gene expression at the post translational level, further contributing to cellular acclimation. By using gene expression as an indicator of changing redox conditions within the ETC, insights into the mechanisms by which photosynthetic organisms perceive and respond genetically to their environment were revealed.

## 1.7 References

Alfonso A, Perewoska I, Kirilovsky D. 2000. Redox Control of *psbA* Gene Expression in the Cyanobacterium *Synechocystis* PCC 6803. Involvement of the Cytochrome $b_6f$ Complex. Plant Physiology 122, 505-515.

Allen JF. 2017. The CoRR hypothesis for genes in organelles. Journal of theoretical biology 434, 50-57.

Ballut L, Marchadier B, Baguet A, Tomasetto C, Séraphin B, Le Hir H. 2005. The exon junction core complex is locked onto RNA by inhibition of eIF4AIII ATPase activity. Nature structural and molecular biology 12, 861.

Baymann F, Brugna M, Mühlenhoff U, Nitschke W. 2001. Daddy, where did PSI come from? Biochemica et Biophysica Acta 1507, 291–310.

Beier D, Gross R. 2006. Regulation of bacterial virulence by two-component systems. Current Opinion in Microbiology 9, 143–152.

Bombar D, Heller P, Sanchez-Baracaldo P, Cater BJ, Zehr JP. 2014. Comparative genomics reveals surprising divergence of two closely related strains of uncultivated UCYN-A cyanobacteria. ISME Journal 8, 2530–2542.

Boulay C, Wilson A, D'Haene S, Kirilovsky D. 2010. Identification of a protein required for recovery of full antenna capacity in OCP-related photoprotective mechanism in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 107, 11620–11625.

Campbell D, Hurry V, Clarke AK, Gustafsson P, Oquist G. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiology and Molecular Biology Reviews 62, 667–683.

Capra EJ, Laub MT. 2012. Evolution of two-component signal transduction systems. Annual Review of Microbiology 66, 325–347.

Chamot D, Colvin KR, Kujat-Choy SL, Owttrim GW. 2005. RNA structural rearrangement via unwinding and annealing by the cyanobacterial RNA helicase, CrhR. Journal of Biological Chemistry 280, 2036–2044.

Chen W, Fang L, Huang X, Ge H, Wang J, Wang X, Zhang Y, Sui N, Xu W, and Wang Y. 2018. Systematic identification of light-regulated cold-responsive proteome in a model cyanobacterium. Journal of Proteomics 179, 100–109.

Chitnis VP, Xu Q, Yu L, Golbeck JH, Nakamoto H, Xie DL, Chitnis PR. 1993. Targeted inactivation of the gene *psaL* encoding a subunit of photosystem I of the cyanobacterium *Synechocystis* sp. PCC 6803. Journal of Biological Chemistry 268, 11678–11684.

Cordin O, Tanner NK, Doère M, Linder P, Banroques J. 2004. The newly discovered Q motif of DEAD-box RNA helicases regulates RNA-binding and helicase activity. The EMBO journal 23, 2478-2487.

Dagan T, Roettger M, Stucken K, Landan G, Koch R, Major P, Gould SB, Goremykin VV, Rippka R, Tandeau de Marsac N, Gugger M, Lockhart PJ, Allen JF, Brune I, Maus I, Pühler A, Martin WF. 2013. Genomes of Stigonematalean cyanobacteria (subsection V) and the evolution of oxygenic photosynthesis from prokaryotes to plastids. Genome Biology and Evolution 5, 31–44.

Enami I, Okumura A, Nagao R, Suzuki T, Iwai M, Shen J-R. 2008. Structures and functions of the extrinsic proteins of photosystem II from different species. Photosynthesis Research 98, 349–363.

Ermakova-Gerdes S, Vermaas W. 1998. Mobility of the primary electron-accepting plastoquinone QA of photosystem II in a *Synechocystis* sp. PCC 6803 strain carrying mutations in the D2 protein. Biochemistry 37, 11569-11578.

Fairman-Williams, ME, Guenther UP, Jankowsky E. 2010. SF1 and SF2 helicases: family matters. Current opinion in structural biology, 20, 313-324.

Fu HY, Picot D, Choquet Y, Longatte G, Sayegh A, Delacotte J, Wollman FA. 2017. Redesigning the $Q_A$ binding site of Photosystem II allows reduction of exogenous quinones. Nature communications 8, 15274.

Fufezan C, Gross CM, Sjödin M, Rutherford AW, Krieger-Liszkay A, Kirilovsky D. 2007. Influence of the redox potential of the primary quinone electron acceptor on photoinhibition in photosystem II. Journal of Biological Chemistry 282, 12492-12502.

García-Domínguez M, Muro-Pastor MI, Reyes JC, Florencio FJ. 2000. Light-dependent regulation of cyanobacterial phytochrome expression. Journal of Bacteriology, 182, 38-44.

Georg J, Rosana ARR, Chamot D, Migur A, Hess WR, Owttrim GW. 2019. Inactivation of the RNA helicase CrhR impacts a specific subset of the transcriptome in the cyanobacterium *Synechocystis* sp. PCC 6803. RNA Biology 16, 1205–1214.

Govindjee and Shevela D. 2011. Adventures with cyanobacteria: a personal perspective. Frontiers in Plant Science 2, 28.

Grossman AR, Schaefer MR, Chiang GG, Collier JL. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiology Reviews. 57, 725– 749.

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A, Saenger W. 2009. Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. Nature Structural & Molecular Biology 16, 334–342.

Hihara Y, Sonoike K, Kanehisa M, Ikeuchi M. 2003. DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. Journal of bacteriology 183, 1719-1725.

Hirano M, Satoh K, Katoh, S. 1980. Plastoquinone as a common link between photosynthesis and respiration in blue-green alga. Photosynthesis Research 1,149–162.

Howitt CA, and Vermaas WFJ. 1998. Quinol and cytochrome oxidases in the cyanobacterium *Synechocystis* sp. PCC 6803. Biochemistry. 37, 17944–17951.

Howitt CA, Cooley JW, Wiskich JT, Vermaas WF. 2001. A strain of *Synechocystis* sp. PCC 6803 without photosynthetic oxygen evolution and respiratory oxygen consumption: implications for the study of cyclic photosynthetic electron transport. Planta 214, 46–56.

Hsiao HY, He Q, Van Waasbergen LG, Grossman AR. 2004. Control of photosynthetic and high-light-responsive genes by the histidine kinase DspA: negative and positive regulation and interactions between signal transduction pathways. Journal of bacteriology 186, 3882-3888.

Huang JY, Chiu YF, Ortega JM, Wang HT, Tseng TS, Ke S, Chu HA. 2016. Mutations of Cytochrome b 559 and PsbJ on and near the QC Site in Photosystem II Influence the Regulation of Short-Term Light Response and Photosynthetic Growth of the Cyanobacterium *Synechocystis* sp. PCC 6803. Biochemistry 55, 2214-2226.

Hubschmann T, Yamamoto H, Gieler T, Murata N, Borner T. 2005. Red and far- red light alter the transcript profile in the cyanobacterium *Synechocystis* sp PCC 6803: impact of cyanobacterial phytochromes. FEBS Letters 579, 1613–1618.

Hurt E and Hauska G. 1981. A cytochrome $b_6f$ complex of five polypeptides with plastoquinol-plastocyanin-oxidoreductase activity from spinach chloroplasts. European Journal of Biochemistry 117, 591–595.

Ibrahim IM, Puthiyaveetil S, Allen JF. 2016. A two-component regulatory system in transcriptional control of photosystem stoichiometry: Redox-dependent and sodium ion-dependent phosphoryl transfer from cyanobacterial histidine kinase Hik2 to response regulators Rre1 and RppA. Frontiers in Plant Science 12, 196–212.

Jarmoskaite I, Russell R. 2014. RNA helicase proteins as chaperones and remodelers. Annual review of biochemistry 83, 697-725.

Joshua S, Mullineaux CW. 2004. Phycobilisome diffusion is required for light-state transitions in cyanobacteria. Plant Physiolology 135, 2112–2119.

Jung K, Fried L, Behr S, Heermann R. 2012. Histidine kinases and response regulators in networks. Current Opinion in Microbiology 15, 118–124.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda

M, Tabata S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Research 3,109–136.

Kanesaki Y, Yamamoto H, Paithoonrangsarid K, Shoumskaya M, Suzuki I, Hayashi H, Murata N. 2007. Histidine kinases play important roles in the perception and signal transduction of hydrogen peroxide in the cyanobacterium, *Synechocystis* sp. PCC 6803. Plant Journal 49, 313–324.

Kerfeld CA, Kirilovsky, D. 2011. Photoprotection in cyanobacteria: The orange carotenoid protein and energy dissipation. Bioenergetic Processes of Cyanobacteria 312, 395-421.

Kobayashi I, Watanabe S, Kanesaki Y, Shimada T, Yoshikawa H, Tanaka K. 2017. Conserved two-component Hik34-Rre1 module directly activates heat-stress inducible transcription of major chaperone and other genes in *Synechococcus elongatus* PCC 7942. Molecular Microbiology 104, 260-277

Komenda J, Barber J. 1995. Comparison of psbO and psbH deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the QB site and dependent on protein synthesis. Biochemistry, 34, 9625–9631.

Kotajima T, Shiraiwa Y, Suzuki I. 2014. Functional analysis of the N-terminal region of an essential histidine kinase, Hik2, in the cyanobacterium *Synechocystis* sp. PCC 6803. FEMS Microbiology Letters 351, 88–94.

Kujat, SL, Owttrim, GW. 2000. Redox-Regulated RNA Helicase Expression. Plant Physiology 124, 703-714.

Lau N, Matsui M, Abduallah AA. 2015. Cyanobacteria: Photoautotrophic Microbial Factories for the Sustainable Synthesis of Industrial Products. BioMed Research International 8, 754934.

Lea-Smith DJ, Ross N, Zori M, Bendall DS, Dennis JS, Scott SA, Smith AG, Howe CJ. 2013. Thylakoid terminal oxidases are essential for the cyanobacterium *Synechocystis* sp. PCC 6803 to survive rapidly changing light intensities. Plant Physiology 162, 484–495.

Li H, Sherman LA. 2000. A redox-responsive regulator of photosynthesis gene expression in the cyanobacterium *Synechocystis* sp. Strain PCC 6803. Journal of Bacteriology 182, 4268–4277.

Linder P, Jankowsky E. 2011. From unwinding to clamping — the DEAD box RNA helicase family. Nature Reviews Molecular Cell Biology, 12, 505–516.

Luo H, Jackson SA, Fagerlund RD, Summerfield TC, Eaton-Rye JJ. 2014. The importance of the hydrophilic region of PsbL for the plastoquinone electron acceptor complex of Photosystem II. Biochimica et Biophysica Actaf-Bioenergetics, 1837, 1435-1446.

Ma W, Deng Y, Mi H. 2008. Redox of plastoquinone pool regulates the expression and activity of NADPH dehydrogenase supercomplex in *Synechocystis* sp. strain PCC 6803. Current Opinions in Microbiology 56, 189–193.

MacColl R. 1998. Cyanobacterial phycobilisomes. Journal of Structural Biology 124, 311–334

Maksimov EG, Mironov KS, Trofimova MA, Nechaeva NL, Todorenko DA, Klementiev KE, Tsoraev GV, Tyutyaev EV, Zorina AA, Feduraev PV, Allakhverdiev SI, Paschenko VZ, Los DA. 2017. Membrane fluidity controls redox-regulated cold stress responses in cyanobacteria. Photosynthesis Research 133, 215–223.

Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N. 2003. Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis* sp. PCC 6803. Proceedings of the National Academy of Sciences of the United States of America USA 100, 9061–9066.

Martin R, Straub AU, Doebele C, Bohnsack MT. 2013. DExD/H-box RNA helicases in ribosome biogenesis. RNA biology 10, 4-18.

Mikami K, Kanesaki Y, Suzuki I, Murata N. 2002. The histidine kinase Hik33 perceives osmotic stress and cold stress in *Synechocystis* sp PCC 6803. Molecular Microbiology 46, 905–915.

Mironov KS, Sidorov RA, Trofimova MS, Bedbenov VS, Tsydendambaev VD, Allakhverdiev SI., Los DA. 2012. Light-dependent cold-induced fatty acid unsaturation, changes in membrane fluidity, and alterations in gene expression in *Synechocystis*. Biochimica et Biophysica Acta 1817, 1352–1359.

Misumi M, Katoh H, Tomo T, Sonoike K. 2016. Relationship Between Photochemical Quenching and Non-Photochemical Quenching in Six Species of Cyanobacteria Reveals Species Difference in Redox State and Species Commonality in Energy Dissipation. Plant and Cell Physiology 57, 1510–1517.

Mohamed A, Jansson C. 1991. Photosynthetic electron transport controls degradation but not production of psbA transcripts in the cyanobacterium *Synechocystis* 6803. Plant molecular biology 16, 891-897.

Mullineaux CW, Allen JF. 1986. The state 2 transition in the cyanobacterium *Synechococcus* 6301 can be driven by respiratory electron flow into the plastoquinone pool. FEBS Letters. 205, 155-160.

Mullineaux CW, Allen JF. 1990. State 1-State 2 transitions in the cyanobacterium *Synechococcus* 6301 are controlled by the redox state of electron carriers between Photosystems I and II. Photosynthesis Research 23, 297–311.

Murata N, Suzuki I. 2006. Exploitation of genomic sequences in a systematic analysis to access how cyanobacteria sense environmental stress. Journal of Experimental Botany 57, 235–247.

Nelson N, Ben-Shem A. 2004. The complex architecture of oxygenic photosynthesis. Nature Reviews 5, 971–982.

Nixon P, Michoux F, Yu J, Boehm M, Komenda J. 2010. Recent advances in understanding the assembly and repair of photosystem II. Annals of Botany 106, 1–16.

Ogawa T, Misumi M, Sonoike K. 2017. Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions. Photosynthesis Research 133, 63–73.

Owttrim, G.W. 2012. RNA helicases in cyanobacteria: biochemical and molecular approaches. Methods in Enzymology 511, 385–403.

Paik I, Yang S, Choi, G. 2012. Phytochrome regulates translation of mRNA in the cytosol. Proceedings of the National Academy of Sciences of the United States of America USA 109, 335–1340.

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I, et al. 2004. Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. Journal of Biological Chemistry 279, 53078–53086.

Plohnke N, Seidel T, Kahmann U, Rogner M, Schneider D, Rexroth S. 2015. The proteome and lipidome of *Synechocystis* sp. PCC 6803 cells grown under light-activated heterotrophic conditions. Molecular Cell Proteomics 14, 572–584.

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF. 2008. The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. Proceedings of the National Academy of Sciences of the United States of America USA 105, 10061–10066.

Prakash JS, Krishna PS., Sirisha K, Kanesaki Y, Suzuki I, Shivaji S, Murata, N. 2010. An RNA helicase, CrhR, regulates the low-temperature-inducible expression of heat-shock genes groES, groEL1 and groEL2 in *Synechocystis* sp. PCC 6803. Microbiology 156, 442-451

Preston C, Critchley C. 1988. Interaction of electron acceptors with thylakoids from halophytic and non-halophytic species. Photosynthesis Research 16, 187–202.

Roberts AG, Kramer DM. 2001. Inhibitor “double occupancy” in the Q(o) pocket of the chloroplast cytochrome $b_6f$ complex. Biochemistry 40, 13407–13412.

Rocak S, Linder P. 2004. DEAD-box proteins: the driving forces behind RNA metabolism. Nature reviews Molecular Cell Biology 5, 232.

Rosana AR, Chamot D, Owttrim GW. 2012a. Autoregulation of RNA helicase expression in response to temperature stress in *Synechocystis* sp. PCC 6803. PLoS ONE 7, e48683.

Rosana AR, Ventakesh M, Chamot D, Patterson-Fortin LM, Tarassova O, Espie GS, Owttrim GW. 2012b. Inactivation of a low temperature-induced RNA helicase in *Synechocystis* sp. PCC 6803: physiological and morphological consequences. Plant and Cell Physiology 53, 646–658.

Rowland JG, Simon WJ, Prakash JS, Slabas AR. 2011. Proteomics reveals a role for the RNA helicase crhR in the modulation of multiple metabolic pathways during cold acclimation of *Synechocystis* sp. PCC6803. Journal of proteome research, 10, 3674-3689.

Schuurmans RM, Schuurmans JM, Bekker M, Kromkamp JC, Matthijs HCP, Hellingwerf KJ. 2014. The Redox Potential of the Plastoquinone Pool of the Cyanobacterium *Synechocystis* Species Strain PCC 6803 Is under Strict Homeostatic Control. Plant Physiology 165, 463–475.

Schreiber U, Endo T, Mi H, Asada K 1995. Quenching analysis of chlorophyll fluorescence by the saturation pulse method: particular aspects relating to the study of eukaryotic algae and cyanobacteria. Plant Cell Physiology 36, 873–882.

Shoumskaya MA, Paithoonrangsarid K, Kanesaki Y, Los DA, Zinchenko VV, Tanticharoen M, Suzuki I, Murata N. 2005. Identical Hik-Rre systems are involved in perception and transduction of salt signals and hyperosmotic signals but regulate the expression of individual genes to different extents in *Synechocystis*. Journal of Biological Chemistry 280, 21531-8.

Sinetova MA, Los DS. 2016. New insights in cyanobacterial cold stress responses: Genes, sensors, and molecular triggers. Biochimica et Biophysica Acta 1863, 2391–2403.

Sireesha K, Radharani B, Krishna PS, Sreedhar N, Subramanyam R, Mohanty P, Prakash JS. 2012. RNA helicase, CrhR is indispensable for the energy redistribution and the regulation of photosystem stoichiometry at low temperature in *Synechocystis* sp. PCC6803. Biochimica et Biophysica Acta 1817, 1525–1536.

Suzuki I, Los DA, Kanesaki Y, Mikami K, Murata N. 2000. The pathway for perception and transduction of low-temperature signals in Synechocystis. The EMBO Journal. 19, 1327-1334.

Tullberg A, Alexciev K, Pfannschmidt T, Allen JF. 2000. Photosynthetic electron flow regulates transcription of the *psaB* gene in pea (Pisum sativum L.) chloroplasts through the redox state of the plastoquinone pool. Plant and Cell Physiology 41, 1045–1054.

Vinnemeier J, Hagemann M. 1999. Identification of salt-regulated genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803 by subtractive RNA hybridization. Archives of Microbiology 172, 377–386.

# Chapter 2: Evidence for convergent sensing of multiple abiotic stresses in cyanobacteria

## 2.1 Abstract

*Background*: Bacteria routinely utilize two-component signal transduction pathways to sense and alter gene expression in response to environmental cues. While cyanobacteria express numerous two-component systems, these pathways do not regulate all of the genes within many of the identified abiotic stress-induced regulons.

*Methods*: Electron transport inhibitors combined with western analysis and measurement of chlorophyll *a* fluorescent yield, using pulse amplitude modulation fluorometry, were used to detect the effect of a diverse range of abiotic stresses on the redox status of the photosynthetic electron transport chain and the accumulation and degradation of the *Synechocystis* sp. PCC 6803 DEAD box RNA helicase, CrhR.

*Results*: Alterations in CrhR abundance were tightly correlated with the redox poise of the electron transport chain between $Q_A$ and cytochrome $b_6f$, with reduction favoring CrhR accumulation.

*Conclusions*: The results provide evidence for an alternative, convergent sensing mechanism mediated through the redox poise of $Q_B/PQH_2$ that senses multiple, divergent forms of abiotic stress and regulates accumulation of CrhR. The RNA helicase activity of CrhR could then function as a post-translational effector to regulate downstream gene expression.

*General significance*: The potential for a related system in *Staphylococcus aureus* and higher plant chloroplasts suggest convergent sensing mechanisms may be evolutionarily conserved and occur more widely than anticipated.

## 2.2. Introduction

Free-living organisms constantly confront multiple abiotic stresses. The ability to sense and respond to these changes in the environment is crucial for their growth and survival. Bacteria frequently utilize two component sensing-signal transduction systems to identify and respond to changes in the environment (Beier and Gross, 2006; Capra and Laub, 2012). Autophosphorylation of a histidine kinase sensor occurs in response to an environmental stimulus, with phosphoryl transfer to the cognate response regulator that mediates alterations in gene expression. Two-component signal transduction pathways generally respond to a single environmental stimulus; thus bacteria encode numerous systems such that the total number is correlated with the number of environmental variables encountered (Jung *et al*., 2012). Although integration of stress responses can occur through various mechanisms, sensing of multiple stresses by a common sensor is infrequent as crosstalk between a sensor kinase and non-cognate response regulator is restricted at a number of levels (Capra and Laub, 2012; Shen and Fang, 2012; Rowland *et al*., 2014).

Cyanobacteria encode numerous, but variable numbers of two-component signal transduction systems. For instance, the model cyanobacteria *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) encode 131/47 histidine kinase (Hik) sensors and 80/45 response regulators respectively (Murata and Suzuki, 2006; Jung *et al*., 2012). In contrast to other bacterial systems, single two-component systems that regulate the entire cyanobacterial cold, salt and osmotic stress-induced regulons have not been identified (Suzuki *et al*., 2001; Mikami *et al*., 2002; Marin *et al*., 2003). An example is salt stress in which the four non-essential histidine kinases, Hik 10, 16/41, 33 and 34, only regulate 20% of the regulon (Marin *et al*., 2003). In addition, although some of the Hik kinases are associated with multiple stress responses, including Hik 33 (Mikami *et al*., 2002; Marin *et al*., 2003; Tu *et al*., 2004; Murata and Suzuki, 2006) and the essential gene Hik 2 (Kotajima *et al*., 2014), the mechanism by which they sense and integrate divergent stress signals is not clear. Furthermore, extensive microarray and proteomic analysis of Hik and Hik-response regulator stress gene regulons have not identified *crhR* or lipid desaturases as downstream targets of any known cold stress responsive genes (Suzuki *et al*., 2001; Marin *et al*., 2003; Paithoonrangsarid *et al*., 2004; Suzuki *et al*., 2005; Slabas *et*

*al*., 2006; Kanesaki *et al*., 2007). These observations imply that additional sensing mechanisms are associated with abiotic stress responses in *Synechocystis*.

Photosynthetic organisms also perceive changes in their environment through associated changes in the redox poise of the photosynthetic electron transport chain. Multiple sites along the chain have been implicated in sensing/signaling short and long-term acclimation responses to re-establish cellular energy balance that is perturbed by the imposition of abiotic stress. A classic example of redox regulation are state transitions, the light-induced process by which photosynthetic organisms, including cyanobacteria, re-distribute excitation energy between photosystem II (PSII) and photosystem I (PSI) (Mullineaux and Allen, 1990; Trebitsh and Danon, 2001; Dietz and Pfannschmidt 2011; Derks *et al*., 2015; Dietz, 2015; Bode *et al*., 2016; Wilde and Hihara, 2016). Prominent among these sites are the redox state of $Q_A$, the first stable electron acceptor in PSII, the intersystem plastoquinone pool (PQ) and ferredoxin (Fd) on the donor side of PSI. Generation of abiotic stress induced reactive oxygen species (ROS) via the photosynthetic electron transport chain also constitutes an additional level of redox signaling, that collectively form a complex network of redox sensors/signals.

The PQ redox potential regulates expression of a diverse set of genes in cyanobacteria including *crhR*, a DEAD box RNA helicase, *kaiC,* a component of the circadian clock, and the chaperonins *groES*/*groEL* (Kujat and Owttrim, 2000; El Bissati and Kirilovsky, 2001; Ivleva *et al*., 2006; Kim *et al*., 2012; Sireesha *et al*., 2012). Signaling downstream from PQ has been shown to involve the response regulator Rre33 that alters photosynthetic gene expression in *Synechocystis* (Li and Sherman, 2000). Recently, Ibrahim *et al*. (2016) have shown that the response regulators Rre1 and Rre33 rapidly dephosphorylate the Hik2 histidine kinase. The authors suggest that Hik2 is the sensor integrating salt and redox signals by regulating the downstream activity of Rre1 and Rre33 (Fig. 2.1) (Ibrahim *et al*., 2016). Thus, while Hik2 appears to be a potential sensor of the redox poise of PQ, the sensing mechanism that activates the Hik2-Rre1/Rre33 two-component signaling pathway is not clear.

Evidence that the redox poise of the electron transport chain (ETC) between $Q_B$ and cytochrome $b_6f$ regulates *crhR* was provided by the observation that the electron transfer inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU and dibromothymoquinone

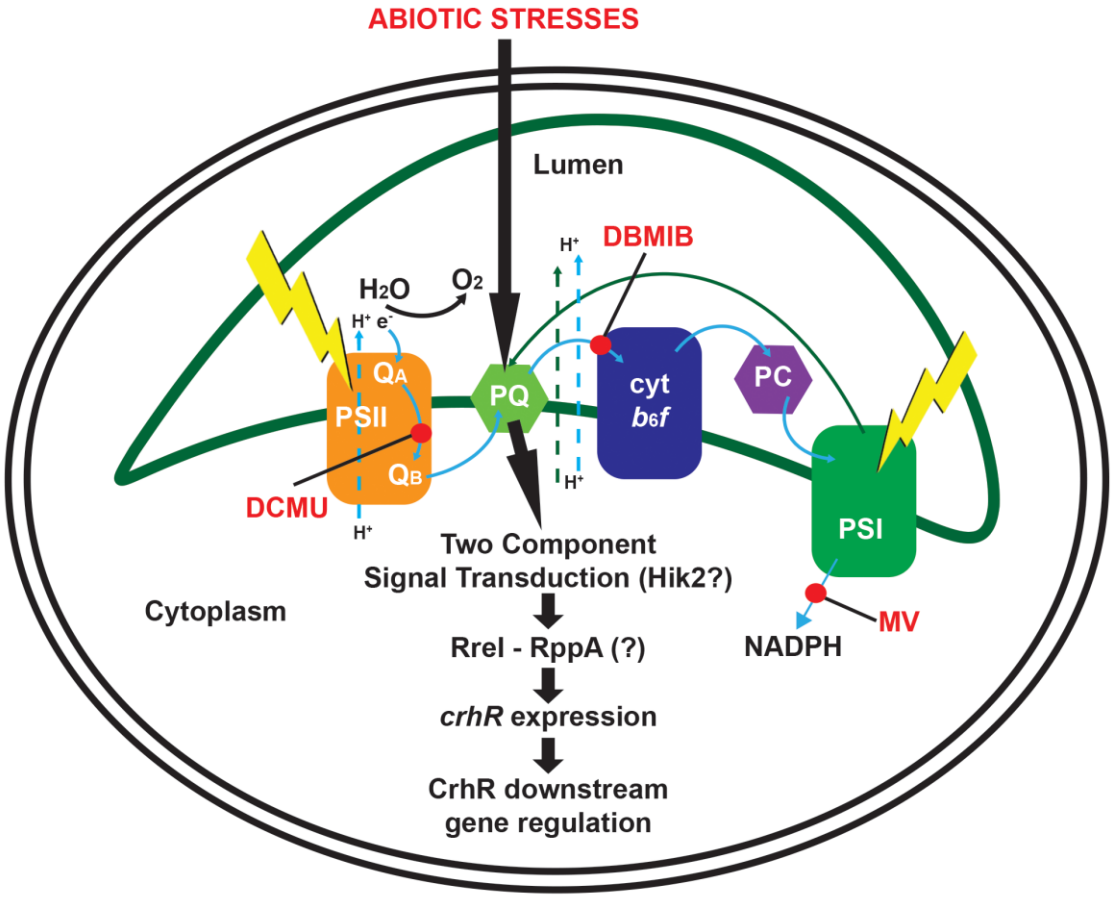



**Fig. 2.1 Overview of the cyanobacterial photosynthetic electron transport chain.** Light energy initially absorbed by phycobilisomes and PSII is used to transfer electrons, ultimately derived from the water-splitting complex, to an electron transport chain. The electrons flow in a linear pathway (blue arrows) from PSII through PQ to cytochrome $b_6f$ into PC and then to PSI. PSI utilizes light energy to transport electrons across the thylakoid membrane to reduce ferredoxin and ultimately $NADP^+$ used for carbon fixation. Linear electron flow also pumps $H^+$ ions into the lumen that are used for the chemiosmotic production of ATP. Electrons can also enter the chain via a cyclic electron transport pathway from PSI to PQ thereby enhancing ATP production at the expense of NADPH (green arrow). PSII, photosystem II; PC, plastocyanin; cyt $b_6f$, cytochrome $b_6f$ complex; PQ, plastoquinone; PSI, photosystem I. Inhibitors that alter electron transport through the chain are indicated in red. DCMU blocks electron transfer from $Q_A$ to $Q_B$ in PSII, DBMIB prevents reduction of cytochrome $b_6f$ from PQ and MV prevents electron transfer from PSI to $NADP^+$. The two-component sensor kinase Hik2 and the response regulators Rre33 and Rre1 have been implicated as players in this signal transduction pathway (Li and Sherman, 2000; Ibrahim *et al*., 2016). Induction of CrhR may further elaborate this signaling cascade by RNA helicase-mediated post-transcriptional regulation of gene expression. Adapted from Berla *et al*. (2015).

(DBMIB) divergently affect expression at 30ºC (Kujat and Owttrim, 2000) (Fig. 2.1). CrhR abundance was significantly diminished in the presence of DCMU, which binds tightly to $Q_B$ and prevents the reduction of PQ, while CrhR expression was relatively unaffected by DBMIB which binds to the $Q_o$ site of cytochrome $b_6f$ and prevents the oxidation of $PQH_2$ (Kujat and Owttrim, 2000). Similarly, in the absence of inhibitors, analysis of transient and steady-state chlorophyll *a* (Chl *a*) fluorescence yield via pulse amplitude modulated fluorometry (PAM) supported the conclusion that *crhR* expression was regulated by the redox poise of PQ (Sireesha *et al*., 2012). In addition, Kujat and Owttrim (2000) showed that *crhR* expression increased above the level detected in cells grown in the presence of glucose in the dark or in the presence of red light that preferentially stimulates PSII in the absence of low temperature stress. Thus, conditions which enhance PQ reduction are directly associated with induction of *crhR* expression.

In the absence of supplied glucose, the glucose tolerant strain of *Synechocystis* used in these studies only obtains energy from light harvesting and electron flow through the ETC. Thus, the PQ redox poise regulation of *crhR* expression is associated with cellular energy homeostasis (Kujat and Owttrim, 2000). In conjunction with light driven electron flow, other abiotic stresses also regulate *crhR* expression. Temperature dramatically affects *crhR* expression, downshift from 30 to 20ºC enhances CrhR abundance by 10-15 fold (Rosana *et al*., 2012a; Sireesha *et al*., 2012) while subsequent upshift to 30ºC induces rapid temperature-dependent proteolysis of CrhR (Tarassova *et al*., 2014). In addition, salt stress also regulates *crhR* expression (Vinnemeier and Hagemann, 1999) indicating that *crhR* induction is not specifically linked to temperature stress (Owttrim, 2013). Unexpectedly, temperature regulation of *crhR* expression involves a complex mechanism consisting of a number of CrhR-dependent auto-regulatory steps (Rosana *et al*., 2012a). These mechanisms included alteration of the abundance and stability of both transcript and protein, culminating in the repression of cold induction by conditional proteolysis of CrhR in response to temperature upshift (Tarassova *et al*., 2014). Recent microarray analysis indicates CrhR regulates a subset of the *Synechocystis* RNA repertoire, consisting of both ncRNA and mRNA transcripts (Georg *et al*., 2019). The data indicated that CrhR impacts multiple aspects of RNA metabolism in *Synechocystis* involving post-transcriptional and transcriptional regulation.

CrhR performs a crucial role in low temperature acclimation as *crhR* inactivation generates a severe cold-sensitive phenotype manifested by physiological and morphological effects at 20°C including degraded thylakoid membranes associated with impaired photosynthesis and aggregated, aberrantly shaped carboxysomes that led to the malfunction of the $CO_2$ concentrating system and diminished $CO_2$ fixation (Rosana *et al*., 2012b). These short- and long-term effects are associated with photosynthesis and energy status, again suggesting CrhR performs a crucial role in cellular acclimation to abiotic stress (Rosana *et al*., 2012b). During longer-term acclimation to low temperature, CrhR, is indirectly associated with the regulation of the PSI genes, *psaA* and *psaB*, and adjustment of the stoichiometry between PSI and PSII (Sireesha *et al*., 2012). Thus, although temperature regulation of *crhR* expression is well studied, we predict that induction of CrhR occurs in response to a common effect of additional abiotic stresses on the redox poise of the ETC.

Here we present evidence that CrhR abundance is regulated by multiple, apparently unrelated abiotic stresses that are sensed through a common mechanism, the redox poise around $Q_B/PQH_2$ and not individual two-component signal transduction pathways. The data identify a convergent environmental sensing mechanism by which a free-living organism can rapidly sense and alter gene expression in response to the diverse range of constantly changing conditions experienced in the natural habitat.

## 2.3. Materials and methods

### 2.3.1 *Strains and culture conditions*

The glucose tolerant strain of *Synechocystis* sp. PCC 6803 (Kujat and Owttrim, 2000) was utilized in these experiments and considered wild type. Cells were grown in liquid BG-11 medium in continuous white light and aerated by shaking at 150 rpm and aerated by bubbling with humidified air (Owttrim, 2012). Photosynthetically active radiation (36 μmol $m^{-2}$ $s^{-1}$, 400–700 nm) was provided by a bank of warm-white fluorescent lamps. Experiments utilized cultures with Chl *a* concentration ranging from 4 – 8 μg/mL.

2.3.2 *Stress and inhibitor conditions*

Unless otherwise stated, an aliquot of an illuminated mid-log phase culture grown at 30$^{o}$C in the absence of stress was harvested to provide the CrhR abundance in unstressed, time zero cells. Cultures were subjected to the following stress conditions by immediate transfer of the culture to 20°C for cold stress or addition of the following compounds: salt 600 mM (NaCl), zinc ($ZnCl_2$) and cobalt ($CoCl_2$•$6H_2O$) (20 μM), osmotic stress was induced by sorbitol (600 mM), oxidative stress was induced using hydrogen peroxide ($H_2O_2$) (0.2 and 2 mM), N,N'-dimethyl-4,4'-bipyridinium dichloride (methyl viologen, MV, 20 μM) and Rose Bengal (RB, 10 μM). The electron transport inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 2.5 μM) and dibromothymoquinone (DBMIB, 10 μM) were dissolved in DMSO as described previously (Kujat and Owttrim, 2000) and MV (20 μM) in water. For the inhibitors dissolved in DMSO, DMSO (0.066%) was added to control cultures that were not treated with the inhibitors. The DCMU concentrations used here are lower than typically used in cyanobacteria to ensure that the cells were not irreversibly damaged by the stress treatments. Experiments comparing the effects of diverse inhibitors and/or stresses were performed on culture aliquots obtained from the same initial culture. A minimum of two biological replicates were performed and representative data is shown.

2.3.3 *Protein content*

Protein immunoblot analysis was performed on soluble protein isolated from culture aliquots using glass bead lysis and clarification by centrifugation as previously described (Owttrim, 2012). The Bradford assay (Bio-Rad Canada, Mississauga, ON) was used to quantify protein, using BSA as the standard. Protein (10 μg) was separated by 10% (w/v) SDS–PAGE, proteins transferred to a nitrocellulose membrane (Protran, Amersham Canada, Oakville, ON) and immunoblots (n=2-4) performed using polyclonal anti-CrhR antisera (1:5,000) raised in rabbit as described by Owttrim (2012). The wild type CrhR (55 kDa) peptide was detected using enhanced chemiluminescence (ECL, Clarity Western ECL Substrate, Bio-Rad Canada, Mississauga, ON). Following transfer, gels were stained with colloidal Coomassie Blue G-250 staining as a control for protein loading (Eaton *et al*., 2013).

2.3.4 *Reactive oxygen species (ROS) analysis*

ROS measurements were performed essentially as described by Hakkila *et al*. (2014) using the cell permeable 5'-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA), a widely used fluorogenic probe for the detection of general oxidative stress. CM-$H_2$DCF-DA diffuses into cells, where its acetate groups are removed by intracellular esterases, yielding nonfluorescent CM-$H_2$DCF. Oxidation of CM-$H_2$DCF by ROS results in the formation of the highly fluorescent CM-DCF (5'-chloromethyl-2', 7'-dichlorofluorescein), the fluorescence from which forms the basis of the ROS detection assay. The $H_2$DCF/DCF fluorescence assay has been shown to be a reliable and versatile method when used in cyanobacteria (e.g. Rastogi *et al*., 2010) to detect the relative, intracellular, abundance of ROS. $H_2$DCF is oxidized directly by the hydroxyl radical, ˙OH, and indirectly by a wide range of ROS including singlet oxygen, $^1O_2$, superoxide, $O_2^-$, $H_2O_2$, and the peroxyl ($RO_2$˙), alkoxyl (RO˙) and hydroperoxyl ($H_2O$˙) radicals, through their circuitous formation of ˙OH (Wardman, 2007). We employed three ROS inducers that produce different species or ROS, $H_2O_2$, Rose Bengal (RB) and methyl viologen (MV). While CM-$H_2$DCF responds directly to the hydroxyl radical produced from the reduction of $H_2O_2$. RB induces formation of singlet oxygen that reacts with cellular constituents to form indirectly detected peroxyl radicals. MV accepts electrons from ferrodoxin and transfers them to $O_2$ yielding the superoxide radical. Superoxide is detected indirectly by CM-$H_2$DCF via its conversion to $H_2O_2$ and reduction to ˙OH . Cell cultures were grown as described, then normalized to an absorbance at 750 nm (A750 nm) = 1. Cells were preloaded with CM-H2DCFDA (5 or 25 µM) for 90 min in the dark at 30 ºC, followed by exposure to the indicated stresses for 15 min or 1 h. The activated DCF dye was excited using 485 nm light and fluorescence at 535 nm detected using a BioTek Instruments Synergy HT (Winooski, VT) fluorescent plate reader. Raw fluorescent values were corrected for autofluorescence at 645 nm. Measurements were performed in triplicate with three biological replicates. Statistical significance was determined via a Student's T-Test using the Graphpad Prism 8 software version 8.0.1, n=3, $p< 0.05$ (GraphPad Software, Inc., La Jolla, CA, USA). Similar results were obtained when using CM-$H_2$DCFDA at 5 µM and 25 µM.

2.3.5 *Chl a fluorescence analysis*

The effect of abiotic stress on the redox potential of the photosynthetic electron transport chain was assessed through the analysis of chlorophyll fluorescence parameters (Ogawa *et al*., 2017). Chlorophyll fluorescence was measured using a Daul-PAM-100 pulse amplitude modulated fluorometer (Heinz Walz GmbH, Effletrich, Germany) (Schreiber, 1986), employing methods that address the unique physiological characteristics of cyanobacteria (Campbell *et al*., 1998; Schuurmans *et al*., 2015; Ogawa *et al*., 2017; Schuurmans *et al*., 2017). At defined periods during the indicated abiotic stress treatment, a sub-sample of the cell suspension (2.7 mL, 4 – 8 µg $mL^{-1}$ Chl *a*) was transferred to a temperature-controlled chamber (Hansatech electrode chamber, Norfolk UK) with magnetic stirring. The measuring head of the PAM fibre optic bundle was subsequently submerged into the suspension from the top of the chamber leaving no headspace, while the other end was connected to the emitter-detector unit. Fluorescence levels were recorded using the supplied data acquisition software v1.19 installed on a computer connected to the Dual-PAM-100. Prior to measurements the cells were dark-adapted for 5 min and then exposed for 2 min to the weak modulated measuring light (620 nm) at 7 µmol photons $m^{-2}$ $s^{-1}$ to determine the minimum fluorescence level, $F_o$, in which all PSII reaction centers are open. The provision of supplemental blue-light (Ogawa *et al*., 2017) had no effect on the level of $F_o$. Due to respiratory electron flow into the common PQ pool in cyanobacteria, however, this procedure does not yield the "true" $F_o$, which was corrected by the formula: $F_{o'} = F_o / ((F_v / F_m) + (F_o / F_s))$ (Oxborough and Baker, 1997). This calculated value for $F_{o'}$ was used in subsequent calculations. A brief, saturating white light pulse (2000 µmol photons $m^{-2}$ $s^{-1}$, 600 ms) was then provided to close all reaction centers and to elicit the maximum available fluorescence yield, $F_{m'}$ in the dark-adapted state. Actinic light (635 nm) to drive photosynthesis was provided at 50 and then 250 µmol photons $m^{-2}$ $s^{-1}$, and the steady state fluorescence yield, $F_s$, was recorded. The fluorescence parameters collected at 250 µmol photons $m^{-2}$ $s^{-1}$ were used for further analysis. During actinic illumination a saturating light pulse was provided at 1 min intervals to periodically determine $F_{m'}$ in the light acclimated state. The maximum fluorescence yield, $F_m$, was determined following the addition of DCMU, which blocks electron transport to $Q_B$ and results in complete

reduction of the primary electron acceptor $Q_A$. The maximum quantum yield of PSII, $\Phi_{PSII}$, was determined from the formula $(F_{m'} - F_s) / F_{m'}$. The fraction of open PSII reaction centers, qP, available to perform photochemistry was calculated as $(F_{m'} - F_s) / (F_{m'} / F_{o'})$, while the fraction of closed reaction centers was estimated as 1 - qP. The non-photochemical quenching parameter $\Phi_{NPQ}$ was calculated as $(F_s / F_{m'}) - (F_s / F_m)$, and non-regulated energy dissipation, $\Phi_{f, D}$, as $F_s / F_m$ (Genty *et al*., 1989; Campbell *et al*., 1998; Ogawa *et al*., 2013). Statistical significance was determined using Student's t-test in the Graphpad Prism 8 software version 8.0.1, n=3, $p< 0.05$ (GraphPad Software, Inc., La Jolla, CA, USA).

## 2.4 Results

### 2.4.1 *CrhR induction occurs in response to diverse abiotic stresses*

We initially investigated the effect of a range of abiotic stresses on CrhR accumulation at 30ºC i.e. in the absence of temperature stress (Fig. 2.2). Controls performed in the absence of added stresses indicated that CrhR is expressed at a low, basal level at 30ºC, a level that at times was below the exposure threshold (compare Fig. 2 & others). At the optimum growth temperature (30ºC), salt stress progressively increased CrhR accumulation over time (Fig. 2.2A) with a response threshold concentration between 100 and 200 mM NaCl (Fig. 2.2B). Salt-stress-induced CrhR accumulation persisted at a high level for up to 6 h then decreased over the next 6 h (Fig. 2.2A), presumably as a result of cellular acclimation. ImageJ analysis of protein abundance (Schneider *et al*., 2012) indicated that CrhR concentration increased linearly for up to three hours following salt stress then plateaued and subsequently decreased at longer exposures (Fig. 2.2A), eventually returning to near-control levels after 24 h (data not shown). The salt-induced accumulation of CrhR could also be readily reversed within a few hours of removing salt from the growth medium (Fig. 2.2C), indicating a dynamic and regulated response of metabolically active cells to the imposition and removal of salt stress. Similar to salt stress, sorbitol-induced osmotic stress increased CrhR accumulation with a threshold between 200 and 300 mM at 30ºC (Fig. 2.2D). A time course following imposition of sorbitol stress (600 mM), revealed an increase in CrhR abundance within 1 h of exposure that progressively increased over time (Fig. 2.2E). Similar profiles of enhanced CrhR

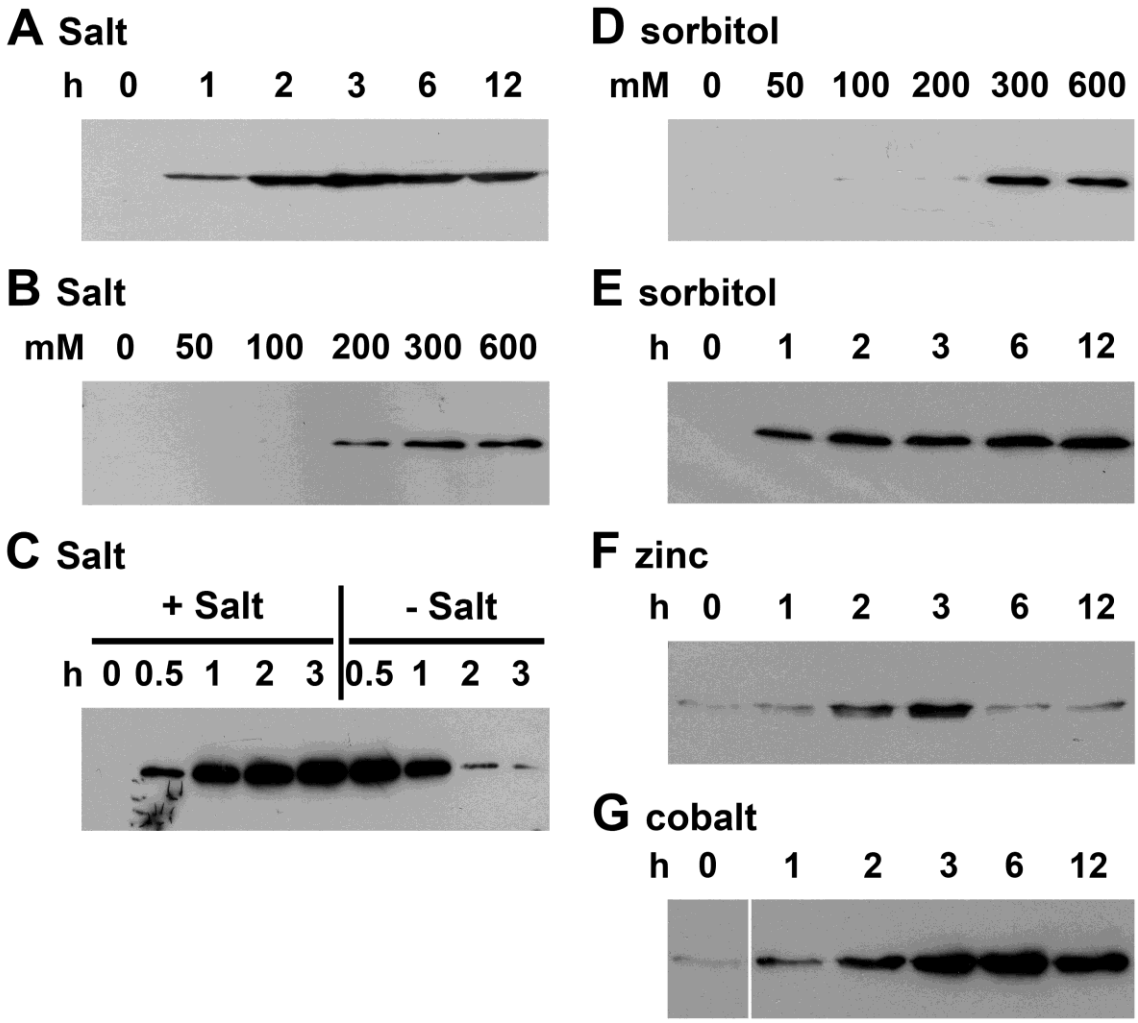



**Fig. 2.2 Effect of abiotic stresses on CrhR abundance.** *Synechocystis* was grown to mid-log phase at 30ºC, exposed to the indicated stress conditions and CrhR abundance was determined by western blot analysis. (A) Salt time course. Wild type cells were exposed to salt stress (600 mM) for the indicated times. (B) Effect of salt concentration. Wild type cells were exposed to the indicated level of salt stress (0-600 mM) for 3 h. (C) Salt induction is reversible. Wild type cells were exposed to salt stress (600 mM) for 3 h at 30ºC, washed and suspended in fresh BG-11 growth media for 3 h at 30ºC. (D) Sorbitol concentration curve. Cells were subjected to osmotic stress with sorbitol at the indicated concentrations for 1 h. (E) Sorbitol time course. Cells were stressed with sorbitol (600 mM) for the indicated times. (F) Zinc and (G) cobalt time courses. Cells were stressed with zinc or cobalt (20 μM) for the indicated times. The same zero time, untreated control was used for both zinc and cobalt.

abundance were also observed in the presence of the heavy metals zinc and cobalt (Fig. 2.2F, G). Both metals produced a transient increase in CrhR expression, with zinc exhibiting a more rapid decrease in CrhR abundance (Fig. 2.2F, G), similar to the salt response (Fig. 2.2A). Thus, four diverse abiotic stresses all elicit a significant enhancement in CrhR accumulation above the basal level observed at 30ºC.

2.4.2 *Reactive oxygen species are not the primary source of CrhR regulation*

Since many of the tested abiotic stresses would also be expected to generate ROS, CrhR accumulation was determined in the absence or presence of the robust, artificial ROS-inducing agents, $H_2O_2$, MV and Rose Bengal (RB) both in the absence and presence of temperature stress. In order to compare the effect of these known ROS inducers, we show the rapid and linear induction of CrhR in response to temperature downshift from 30 to 20ºC (Fig. 2.3A), similar to the previous induction by salt, sorbitol, $Zn^{2+}$ or $Co^{+2}$ stress (Fig. 2.2A, E-G). In contrast, exposure to $H_2O_2$ at 30ºC resulted in negligible induction of CrhR after 45 min and marginal accumulation after 1 h (Fig. 2.3B). MV and RB treatment generated similar results, minimal CrhR induction after 1 h of exposure at 30ºC (Fig. 2.3C, D). This minimal effect, however, was not maintained as CrhR decreased over the next 2 h (Fig. 2.3C, D), unlike the induction pattern observed for abiotic stresses. Furthermore, MV and RB did not affect the subsequent time course or magnitude of the low temperature induction of CrhR (Fig. 2.3C, D). Analysis of the MV effect on repression of induction indicated that MV did not affect CrhR induction at 20ºC (Fig. 2.3E, F). Interestingly, the anticipated repression by temperature upshift to 30ºC was not observed in the presence of MV (Fig. 2.3E) while repression was observed in the presence of RB (Fig. 2.3F), suggesting that perhaps the MV effect on the ETC delayed or curtailed repression.

Fluorometric detection of ROS was also conducted to determine if there was a consistent correlation between the stress-induced level of CrhR accumulation and ROS generation. As shown in Fig. 2.4, *Synechocystis* exposure to stresses shown to significantly induce CrhR accumulation, including $CoCl_2$ and cold shock (Figs. 2.2G, 2.3A), did not result in significant ROS production (Fig. 2.4). Conversely, while known photosensitizers

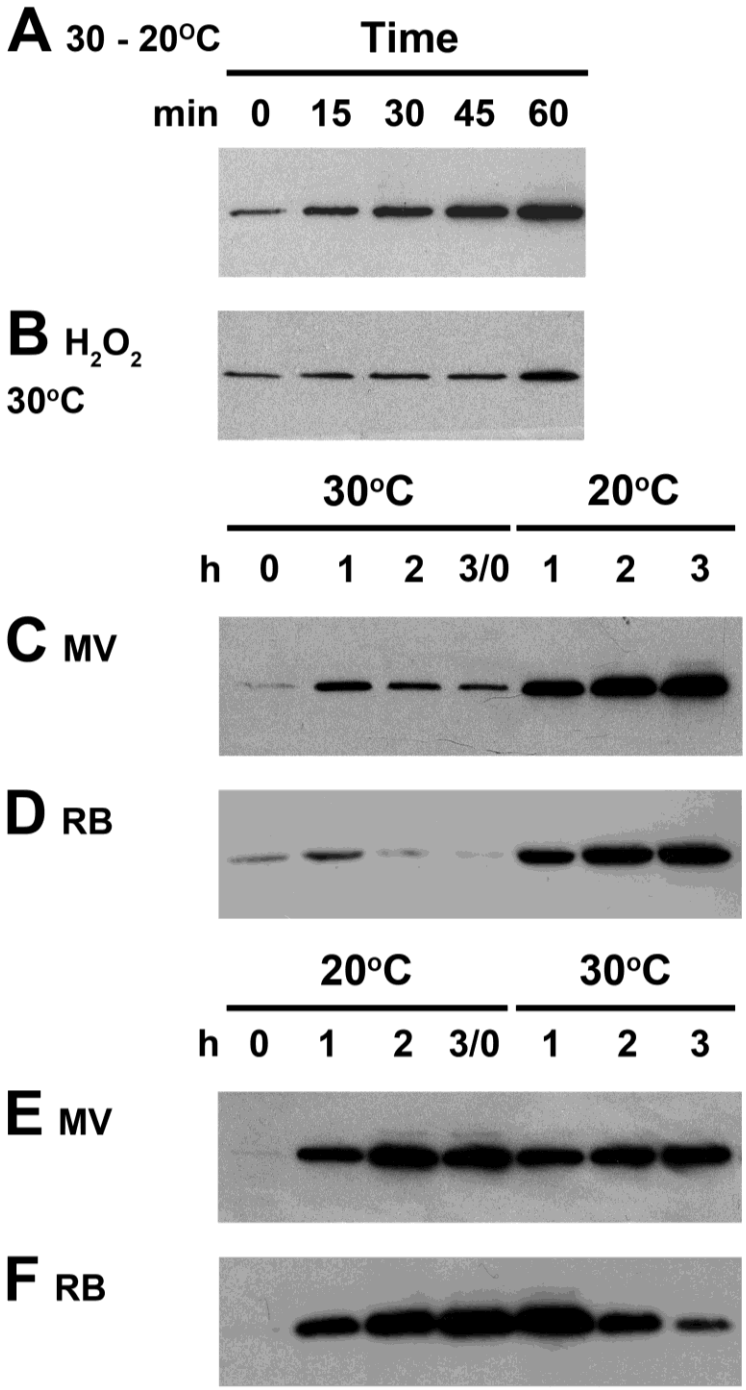



**Fig. 2.3 Oxidative stress effect on CrhR accumulation.** Wild type *Synechocystis* cells were grown to mid-log phase at 30°C and exposed to the indicated stresses. (A) Temperature downshift. A downshift in temperature from 30 to 20°C for 1 h was included as a control for the level of CrhR induction routinely observed for low temperature stress. Oxidative stress was induced by exposure to the following compounds: (B) $H_2O_2$ (200 µM) at 30°C for 1 h, (C) MV (20 µM) and (D) Rose Bengal (RB, 10 µM) at 30°C and in combination with temperature downshift from 30 to 20°C and (E) MV and (F) RB in combination with temperature downshift from 30 to 20°C followed by temperature upshift from 20°C to 30°C.

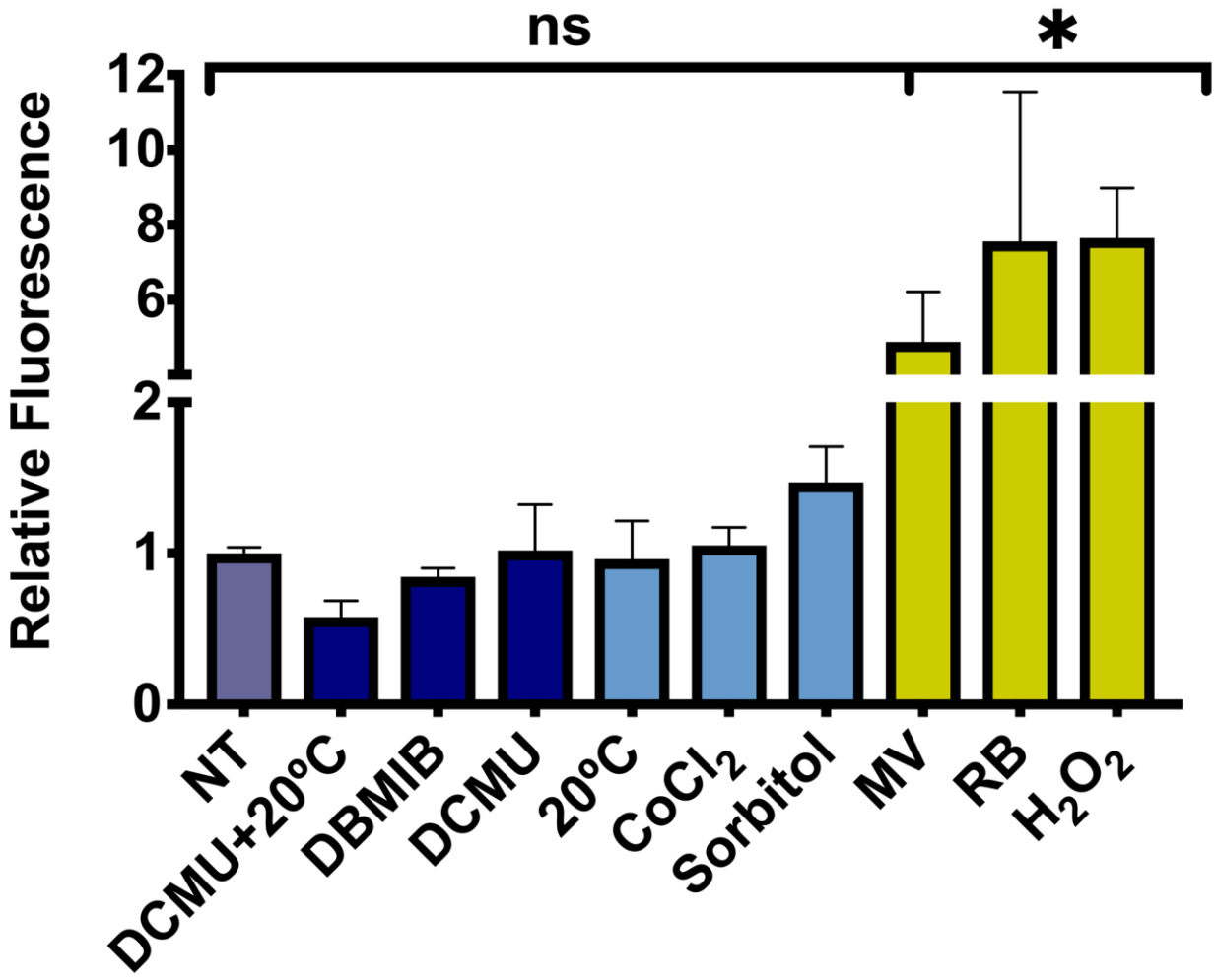



**Fig. 2.4 Abiotic stress effect on ROS abundance.** *Synechocystis* cultures were adjusted to $A_{750nm}$ = 1 and dark incubated for 90 min at 30 ºC in the presence of the CM-$H_2$DCFDA fluorescent dye (5 µM) before exposure to the indicated stresses for 1 h. All stressed cells were incubated under standard light conditions. Purple, non-treated (NT) wild type control; dark blue, ETC inhibitor; light blue, abiotic stress treatment; yellow, ROS inducer treatment. Bars display the average of n = 3 to 7 biological replicates ± standard deviation relative to untreated cells at 30 ºC. Asterisks denote the significance significance as determined by Student's t-test: *=P<0.05. ns, not significant.

including $H_2O_2$, MV and RB artificially generated ROS to notably higher levels (Fig. 2.4), CrhR induction was minimal under these conditions (Fig. 2.3B-F). Together the data suggest that stress induction of CrhR is not directly correlated with ROS abundance.

*2.4.3 Redox status of $Q_B$/$PQH_2$ is a primary source of CrhR regulation*

The influence of the redox state of the photosynthetic electron transport chain on the induction of CrhR by abiotic stresses was assessed in the presence of DCMU or DBMIB (Fig. 2.5). These electron transport inhibitors have opposite effects on $Q_B$/$PQH_2$; being more oxidized in the presence of DCMU and more reduced in the presence of DBMIB (Trebst, 1980). However, both inhibitors affect ETC components downstream of cytochrome *$b_6f$* similarly, leading to oxidation of PSI and Fd. In comparison to untreated controls (Fig. 2.5 A, D, G), DCMU (2.5 μM) significantly inhibited CrhR accumulation under all three representative and divergent stresses tested: low temperature, high salt and heavy metal stress (Fig. 2.5B, E, H). In contrast, the kinetics and magnitude of stress-induced CrhR accumulation was unaffected or slightly enhanced by the presence of DBMIB (Fig. 2.5C, F, I). Notably, the DCMU and DBMIB results were also obtained in the absence of significant ROS production (Fig. 2.4), suggesting again that ROS was a minor contributor to abiotic stress-induced CrhR accumulation.

The results presented in Fig. 2.5 imply that the stress induced increase in CrhR abundance occurs through effects on the redox potential of the ETC upstream beyond $Q_A$ but before cytochrome *$b_6f$*, most likely $Q_B$/$PQH_2$. In order to determine if the redox potential downstream of cytochrome *$b_6f$* played a role in CrhR accumulation, we examined the effect of combinations of the three ETC inhibitors on CrhR abundance. MV oxidizes the donor side of PSI, and thus also PQ and Fd, allowing the potential contribution of the redox potential of Fd, as well as cyclic electron flow around PSI on CrhR abundance to be determined. Simultaneous addition of MV and DCMU essentially negated CrhR expression/induction in response to both temperature upshift and downshift (Fig. 2.6A, B), similar to the results observed with DCMU alone (Fig. 2.5B). Similarly, a combination of all three inhibitors resulted in limited induction of CrhR at 20ºC while repression was still observed at 30ºC (Fig. 2.6C). A time course of CrhR accumulation in the presence of DCMU and DBMIB detected basal levels of CrhR accumulation, indicating that some

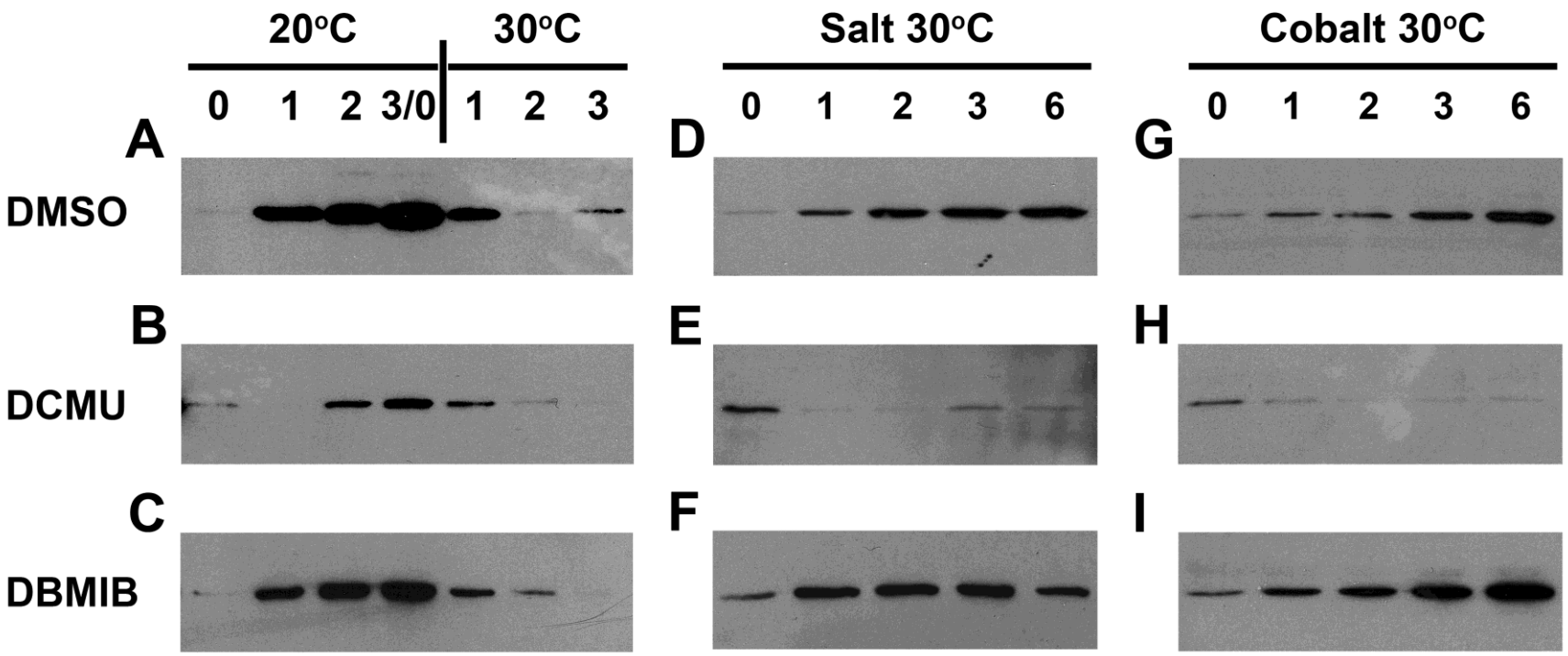



**Fig. 2.5 Abiotic stress induction of CrhR in the presence of ETC inhibitors.** Wild type *Synechocystis* cells were grown to mid-log phase at 30°C and exposed to the selected abiotic stress conditions in combination with either DCMU or DBMIB. (A-C) Temperature shift. Cells grown at 30°C were transferred to 20°C for 3 h and then returned to 30°C for 3 h in the (A) absence or (B) presence of DCMU or (C) DBMIB. (D-F) Salt induction of CrhR. Cells grown at 30°C were subjected to salt stress (600 mM) for the indicated times in the (D) absence or (E) presence of DCMU or (F) DBMIB. (G-I) Cobalt induction of CrhR. Cells grown at 30°C were subjected to cobalt stress (20 μM) for the indicated times in the (G) absence or (H) presence of DCMU or (I) DBMIB. Simultaneous experiments were performed in the presence of DMSO as the inhibitor solvent control, DCMU (2.5 μM) or DBMIB (10 μM)

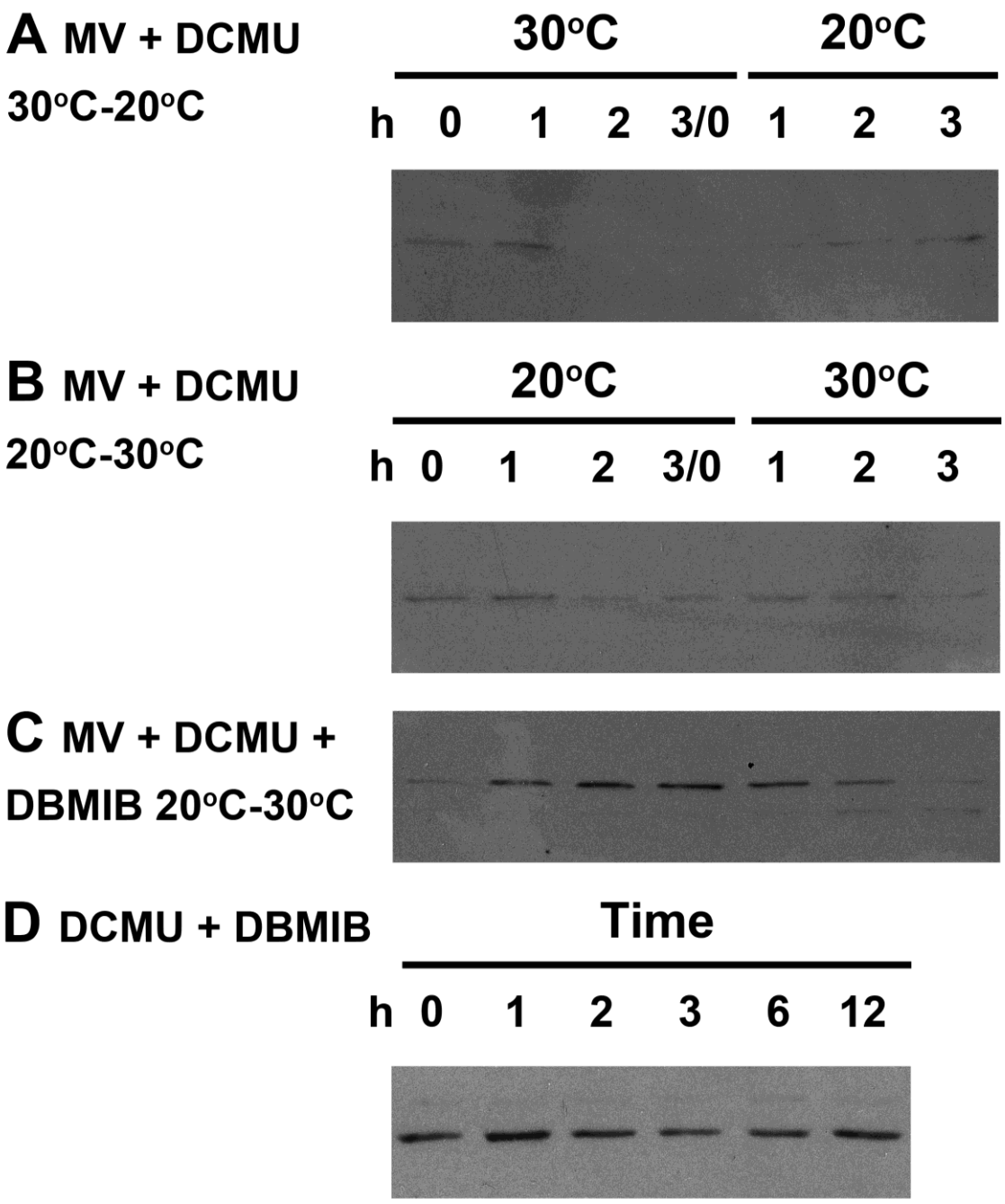



**Fig. 2.6. Combinatorial effect of electron transport inhibitors on CrhR abundance.** Wild type *Synechocystis* cells were grown to mid-log phase at 30ºC and the effect of temperature shift analyzed in conjunction with the indicated combinations of ETC inhibitors. (A) Temperature downshift. The effect of MV + DCMU on CrhR accumulation at 30ºC followed by downshift to 20ºC. (B-C) Temperature upshift. The effect of the inhibitor combinations (B) MV + DCMU and (C) MV + DCMU + DBMIB on CrhR accumulation at 20ºC followed by upshift to 30ºC. (D) DCMU + DBMIB time course. Time course of DCMU + DBMIB effect on CrhR accumulation at 30ºC. Inhibitor concentrations: MV (20 μM), DCMU (2.5 μM) and DBMIB (10 μM).

electrons were flowing into the ETC, most likely via cyclic electron transport (Figs. 2.1, 2.6D). Overall, these results demonstrate that for CrhR, the redox potential upstream of cytochrome *$b_6f$* and not Fd is the primary determinant of CrhR abundance.

2.4.4 *Activity of the electron transport chain varies in response to abiotic stress*

The common effect of each stress on CrhR accumulation was further linked to the ETC redox poise by measurements of Chl *a* fluorescence yield determined using a pulse amplitude modulation (PAM) fluorometer. Fig. 2.7A shows a typical, annotated example of a Chl *a* fluorescence trace of *Synechocystis* at 30°C (no treatment, NT) indicating the measured parameters $F_o$, $F_m$, $F_s$ and $F_{m'}$, as used in this study. Initially we examined the effect of electron transport inhibitors and ROS inducers on PAM fluorescence. Both DCMU, which causes maximal $Q_A$ reduction, and DBMIB, which prevents reduction of cytochrome *$b_6f$*, caused a rise in fluorescence yield (Fig. 2.7A, B). A similar increase in fluorescence yield was not observed in the presence of either MV, which affects PSI redox poise and generates ROS, or $H_2O_2$ that induces ROS stress (Fig. 2.7C, D).

In the absence of inhibitors and ROS inducers, an increase in actinic light from 50 to 250 µmol photons $m^{-2}$ $s^{-1}$ caused a rise in $F_s$, as anticipated for photosynthetically active cells (Fig. 2.7A). Under this condition, approximately 58% of PSII reaction centers were open and available for photochemistry as indicated by the photochemical quenching parameter qP (Fig. 2.8A). At constant light, a statistically significant decrease in photochemically available PSII reaction centers occurred in the presence of the various abiotic stresses indicating a stress-associated closure of PSII (1- qP) and enhanced reduction of $Q_A$ and the PQ pool (Fig. 2.8A). Notably, stress effects on qP clustered together and were significantly different from those observed for ROS inducers or stress levels which did not induce CrhR accumulation (Fig. 2.8A). The effect of the low temperature (20 °C), salt, osmotic and heavy metal stress on qP are not transient, but are rapid and maintained throughout the three hour time course of the experiments (Fig. 2.8B–E), in parallel with the time course for maximal CrhR accumulation (Fig. 2.2). In conjunction, the effective quantum yield of PSII ($\Phi_{PSII}$) was also significantly reduced by abiotic stresses that induce CrhR accumulation (Fig. 2.9A). In contrast, oxidative stress elicited by RB and $H_2O_2$ led to marginal and statistically insignificant decreases in qP and

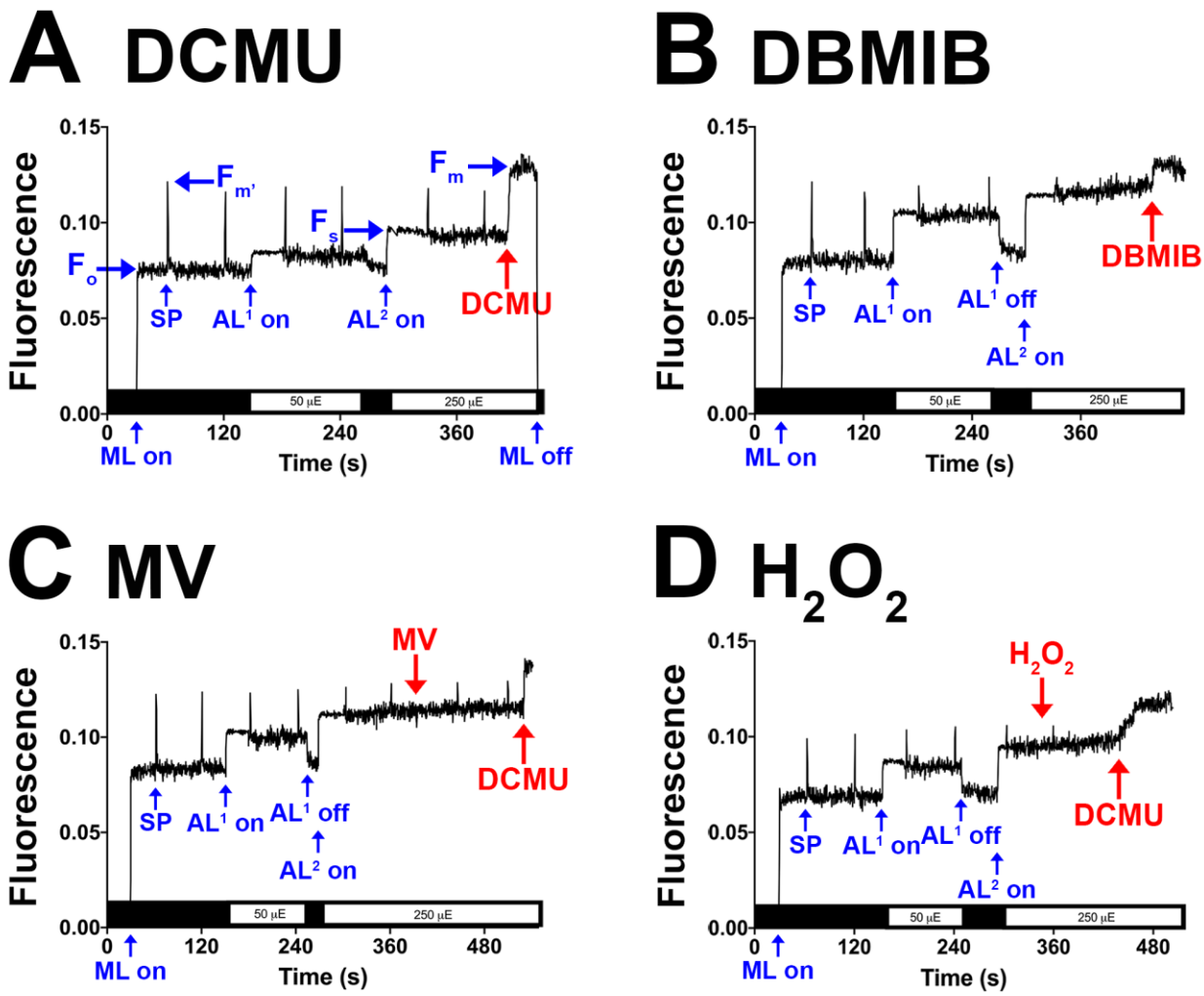



**Fig. 2.7. Electron transport inhibitor and ROS effects on Chl *a* fluorescence yield.** Representative traces generated by PAM fluorometry are shown to illustrate the effect of abiotic stresses on Chl *a* fluorescence. When indicated, DCMU (20 µM), DBMIB (20 µM), MV (20 µM) or $H_2O_2$ (20 µM), were added. (A) DCMU effect. $F_o$ was determined in dark-adapted *Synechocystis* cells grown at 30°C in measuring light. A saturating light pulse generated $F_{m'}$, stable fluorescence, $F_s$, was determined in non-saturating actinic light at both 50 and 250 µmol $m^{-2}$ $s^{-1}$ and maximum fluorescence, $F_m$, was determined in the presence of DCMU. (B-D) Effect of DBMIB, MV and $H_2O_2$. The effect of the indicated ETC inhibitors and/or ROS inducers on fluorescence was determined in *Synechocystis* cells grown at 30°C. ML, measuring light (7 µmol photons $m^{-2}$ $s^{-1}$); $AL^1$ on, actinic light (50 µmol $m^{-2}$ $s^{-1}$); $AL^2$ on, actinic light (250 µmol $m^{-2}$ $s^{-1}$); SP, saturating pulse (2000 µmol $m^{-2}$ $s^{-1}$).

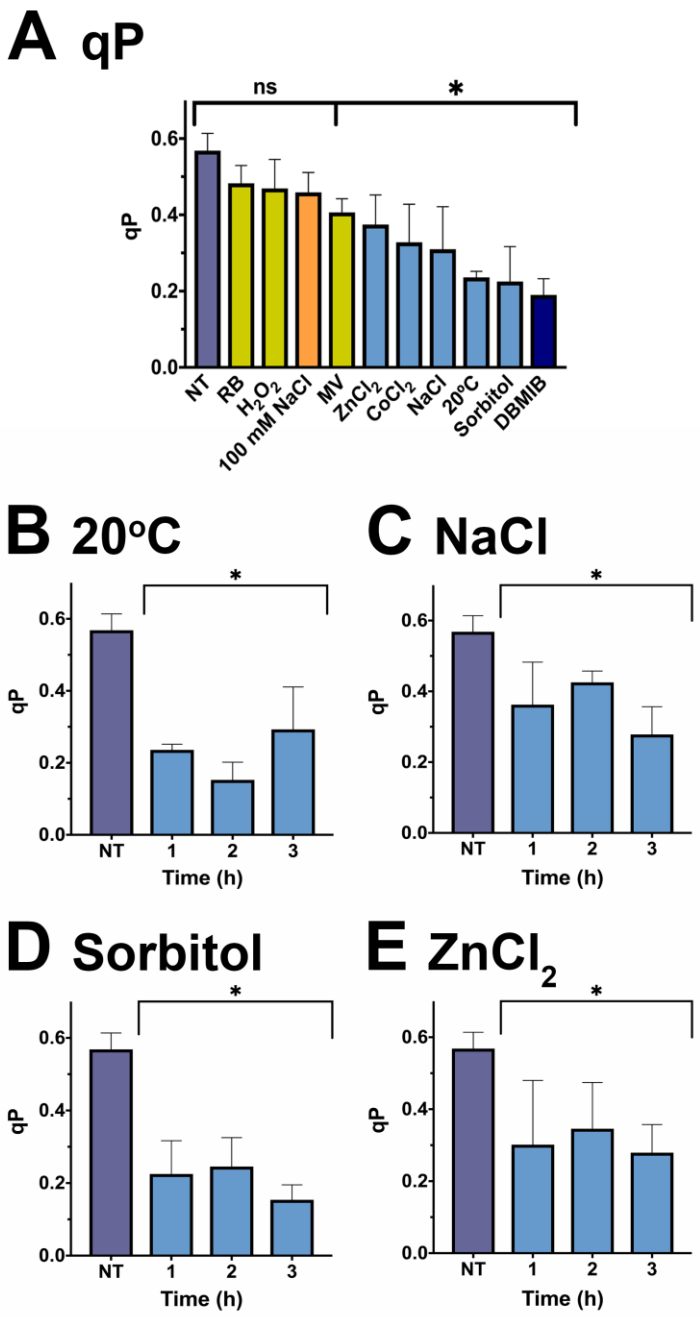



**Fig. 2.8. Effect of stress treatments on the coefficient of photochemical quenching, qP.** (A) Stress effects on qP. qP was determined in a *Synechocystis* culture grown at 30ºC (NT) and after 1 h exposure to the indicated stresses. (B-D) qP time courses. qP was determined in a *Synechocystis* culture grown at 30ºC (NT) and at 1 h intervals after exposure to the indicated stresses, NaCl (600 mM), sorbitol (600 mM) and $ZnCl_2$ (20 µM). Purple, non-treated (NT) wild type control; orange, CrhR non-inducing abiotic stresses; dark blue, ETC inhibitor; light blue, abiotic stress treatment; yellow, ROS induction treatment. Bars display the average of n = 3 biological replicates ± standard deviation relative to untreated cells at 30 ºC. Asterisks denote the statistical significance relative to untreated cells at 30 ºC, as determined by Student's t-test: *=P<0.05. ns, not significant.

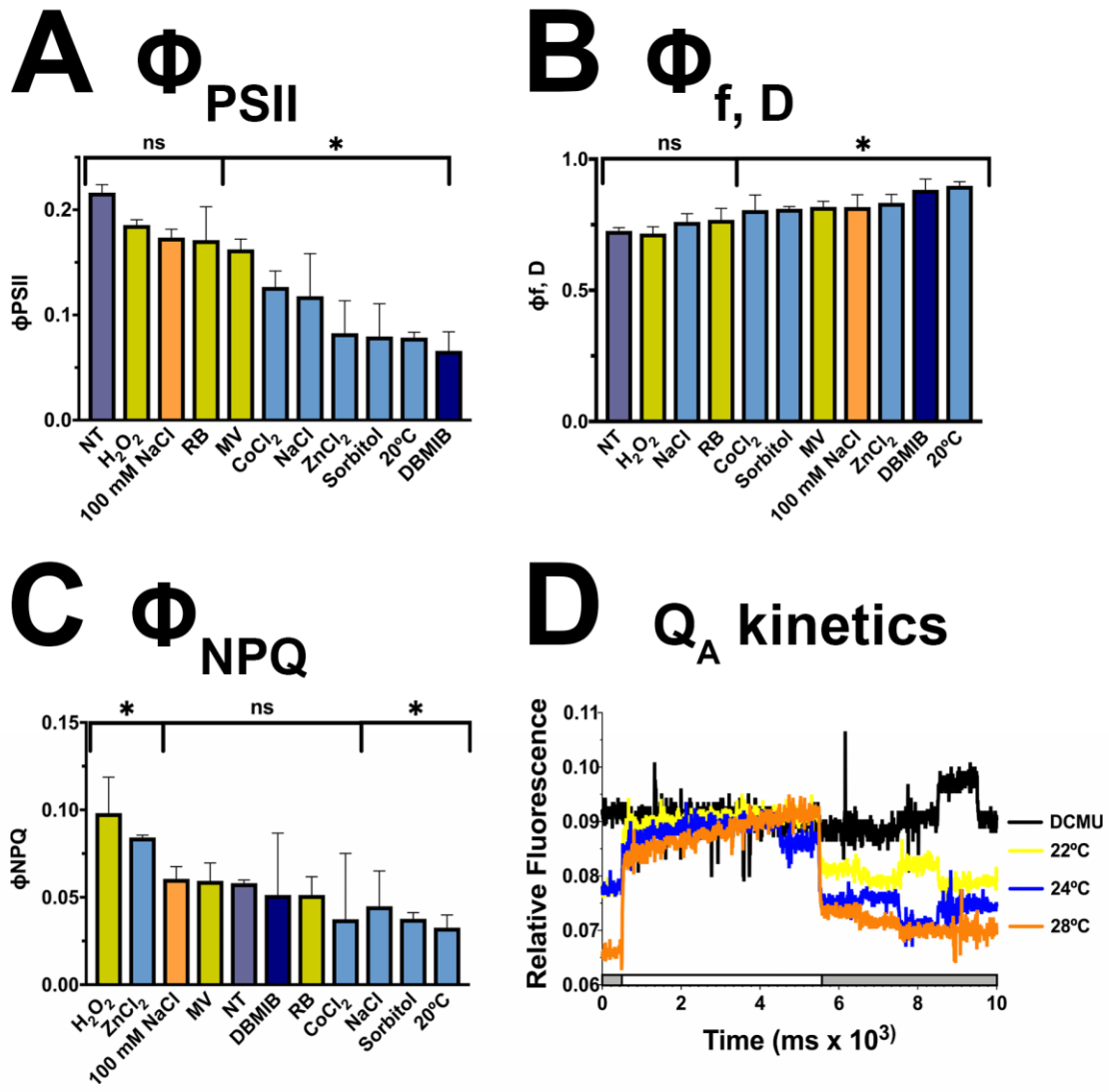



**Fig. 2.9. Treatment effects on fluorescence parameters.** Wild type *Synechocystis* cells were grown to mid-log phase at 30ºC, exposed to the indicated stresses for 1 h and the cells subjected to PAM analysis. (A) $\Phi_{PSII}$, (B) $\Phi_{f, D}$ and (C) $\Phi_{NPQ}$. Purple, non-treated (NT) wild type control; orange, CrhR non-inducing abiotic stresses; dark blue, ETC inhibitor; light blue, abiotic stress treatment; yellow, ROS induction treatment. Asterisks denote the statistical significance relative to untreated cells at 30ºC as determined by Student's t-test: *=P<0.05. ns, not significant. (D) $Q_A$- re-oxidation kinetics. Fluorescent traces illustrating the $Q_A$- re-oxidation kinetics in response to temperature decrease. Fluorescent measurements were initiated on cells incubated at 30ºC in a Clark electrode chamber in actinic light (250 µmol $m^{-2}$ $s^{-1}$, light grey bar). The temperature of the water jacket was adjusted from 30ºC to 20ºC and fluorescent traces recorded after maximum fluorescence was generated by a saturating light pulse (2000 µmol $m^{-2}$ $s^{-1}$ for 600 ms; white bar) followed by $Q_A$- re-oxidation in actinic light (250 µmol $m^{-2}$ $s^{-1}$, light grey bar) as the chamber temperature progressively decreased to 28ºC (orange line), 24ºC (blue line) and 22ºC (yellow line). DCMU (10 µM; black line) was then added to achieve complete $Q_A$ reduction.

$\Phi_{PSII}$, while the impact of MV was intermediate (Figs. 2.8A, 2.9A). Moderate NaCl and osmotic stress, which failed to induce CrhR accumulation (Figs. 2.2 and 2.5), also had marginal effects on the redox state of $Q_A$ and the quantum yield of PSII (Figs. 2.8A, 2.9A). However, a concentration of NaCl sufficient to induce CrhR also resulted in a significant closure of PSII (1- qP), $Q_A$ reduction and decreased $\Phi_{PSII}$ (Figs. 2.8A, 2.9A). Thus, there is a tight correlation between a threshold level of stress-induced decreases in qP and $\Phi_{PSII}$ and the stress-induced accumulation of CrhR.

### 2.4.5 *A role for non-photochemical quenching?*

In addition to driving photochemistry, the energy of absorbed photons may also be dissipated through non-regulated ($\Phi_{f, D}$) and regulated ($\Phi_{NPQ}$) non-photochemical mechanisms, which themselves might also contribute to signal CrhR induction. This did not appear to be the case, as a majority of cells experiencing stress conditions displayed an enhanced pattern of non-regulated energy dissipation (Fig. 2.9B). Conversely, 5 of 9 stress conditions resulted in no significant change in regulated, non-photochemical quenching, $\Phi_{NPQ}$, while the $H_2O_2$ and $ZnCl_2$ treatments led to significantly enhanced $\Phi_{NPQ}$ and the DBMIB and low temperature (20 °C) treatment led to significantly lower $\Phi_{NPQ}$ relative to the control (Fig. 2.9C). Thus, abiotic stresses that lead to CrhR induction or those that induce ROS do not evoke a common pattern of non-regulated or regulated energy dissipation. It appears that each stress affects non-photochemical energy dissipation in its own way. These disparate outcomes suggest that mechanism leading to non-photochemical quenching in cyanobacteria, such as the orange carotenoid protein pathway or state transitions (Misumi *et al*., 2016) are not responsible for induction of CrhR (Fig. 2.9C).

The re-oxidation kinetics of $Q_A^-$ after a saturating light flash in response to a graded temperature downshift is shown in Fig. 2.9D. As the incubation temperature progressively decreased, the rate of electron flow out of $Q_A$ into the PQ pool also progressively decreased, reflective of the rapid attainment of a more reduced $Q_A$ pool under this stress condition and an acceptor side limitation. Thus, this data again show that a temperature gradient from 30ºC to 20ºC results in progressive reduction of the ETC beyond $Q_A$ which directly correlates with enhanced CrhR accumulation.

Collectively, the PAM results indicate that a diverse range of abiotic stresses decrease the overall efficiency of photosynthesis, accompanied by increased ETC reduction surrounding $Q_B$ and the PQ pool that correlate with enhanced CrhR abundance. In contrast, ROS and non-photochemical quenching of Chl *a* fluorescence do not appear to play a significant role in this phenomenon.

## 2.5 Discussion

Here we address a long-standing question regarding the mechanism by which cyanobacteria regulate expression of abiotic stress responsive genes that are not directly controlled by stress-specific two component signal transduction systems. We provide evidence that accumulation of the cyanobacterial RNA helicase, CrhR, a potential stress-responsive downstream effector, is regulated by a convergent sensing mechanism, involving enhanced reduction of the ETC in the vicinity of PQ by a diverse range of abiotic stresses. An important observation was that CrhR expression displayed a consistent pattern of linear induction when confronted with a diverse range of abiotic stresses, suggesting a common mechanism. Notably, CrhR induction occurred in the presence of diverse abiotic stresses at 30ºC indicating that *crhR* is not simply a cold stress gene. Furthermore, repression of the system also occurred in a consistent manner in response to removal of the stress, as previously shown to occur for temperature upshift (Tarassova *et al*., 2014).

### 2.5.1 *Evidence for a convergent sensing mechanism*

Previously, we have shown that preferential stimulation of PSII in the light, exogenous glucose administered in the dark and DBMIB enhance *crhR* expression at 30ºC. In contrast, DCMU substantially decreased *crhR* expression at 30ºC (Kujat and Owttrim, 2000). Here, the critical contribution of the redox poise of the ETC upstream of cytochrome $b_6f$ to the induction of CrhR accumulation was further demonstrated using a variety of inhibitors and growth conditions that alter electron flow through the ETC (Fig. 2.1). In response to all conditions tested, enhanced reduction or oxidation of the ETC stimulated or inhibited CrhR accumulation, respectively, an effect that was reproducibly observed in response to all tested abiotic stresses and was not strictly dependent on low temperature stress. Furthermore, the divergent effects of DCMU and DBMIB indicate that

the redox poise of $Q_A$ does not regulate CrhR accumulation since enhanced reduction of $Q_A$ would occur in the presence of both inhibitors. Overall, the results suggest that CrhR accumulation can be regulated by conditions, either environmental or inhibitor induced, that alter the $PQH_2$:PQ ratio.

Evidence that the redox status of PSI and/or Fd was not responsible for regulation of CrhR was indicated by the divergence of CrhR abundance in the presence of DCMU and DBMIB under conditions where both Fd and PSI would be oxidized. Similarly, MV treatment provided further evidence that the redox status of PSI was not strongly associated with CrhR induction but may play a role in CrhR proteolysis. As MV accepts electrons directly from both PSI and Fd (Sétif, 2015), Fd will be oxidized in its presence and cyclic electron flow will be impaired (Fan *et al*., 2009). However, Nishiyama *et al*. (2001) have shown that MV also has an observable effect on linear electron flow through PQ in *Synechocystis*. We observed this effect as a marginal but transient stimulation of CrhR accumulation at 30ºC in the presence of MV, an effect that rapidly declined with time. Subsequent normal induction of CrhR by temperature downshift in the presence of MV suggests that induction or repression of CrhR were not dependent on Fd or PSI. Furthermore, DCMU superseded the effects of MV and temperature when tested alone or in combination with other ETC inhibitors, while DBMIB treatment partially restored CrhR induction in the presence of DCMU and/or MV. Under these conditions, while DCMU will block electron transfer from $Q_A$ to $Q_B$ and result in a general oxidation of the ETC at and downstream of $Q_B$, DBMIB will enhance reduction of the ETC upstream of cytochrome $b_6f$. It should be noted that although it was reported that DBMIB could affect PQ reduction in spinach chloroplasts and thylakoid membranes (Belatik *et al*., 2013) this does not appear to be the case in *Synechocystis*. More recent analysis indicated that DBMIB, although it can cause minimal oxidation of PSII, this effect was negated by the block in cytochrome $b_6f$ function, resulting in enhanced PQ reduction in cyanobacteria (Calzadilla *et al*., 2019). Collectively, the data from a combination of ETC inhibitor(s) and abiotic stresses suggest that the redox potential of components of the ETC between $Q_A$ and cytochrome $b_6f$ regulate CrhR expression.

Proteolytic repression of CrhR also occurred upon removal of the abiotic stresses within the same 3 h time-scales previously reported in response to temperature upshift,

suggesting that CrhR induction and repression are controlled by divergent pathways (Rosana *et al*., 2012a; Tarassova *et al*., 2014). The proteolytic repression observed upon relief of abiotic stress indicated that there was no major effect on cellular metabolism after stress and/or inhibitor treatment.

### 2.5.2 *ROS-independent induction of* CrhR

Since enhanced reduction of the ETC will naturally increase ROS stress, conditions were utilized to differentiate the contribution of the two processes to CrhR regulation. If ROS was the primary signal regulating CrhR abundance, then ROS generating conditions should correlate with proportionally enhanced CrhR levels. This was not the case, as a range of ROS species resulted in either no induction or significantly less, non-linear accumulation of CrhR compared to the other abiotic stresses. Together, the results suggest that ROS is not directly responsible for CrhR induction, however some ROS generators marginally influence CrhR accumulation in a transient fashion, potentially by secondary effects on the redox poise of the ETC.

### 2.5.3 *Diverse abiotic stresses cause enhanced reduction of the ETC*

Direct evidence for the effect of abiotic stress on the ETC was obtained through PAM fluorometry. In cyanobacteria, the photosynthetic and respiratory electron transport chains (ETC) share common components that function in the same thylakoid membrane (Mullineaux, 2013). Despite issues arising from interaction between respiratory and photosynthetic electron flow, PAM fluorometry has been shown to be a reliable method for the comparative measurement of the redox poise of the electron transport chain within a individual cyanobacterial strain (Campbell *et al*., 1998; Schuurmans *et al*., 2015; Ogawa *et al*., 2017; Schuurmans *et al*., 2017). We utilized both $\Phi_{PSII}$ and qP values as indicators of photosynthetic activity, offering an indirect measurement of the redox poise of the PQ pool (Mullineux and Allen; 1986; Schuurmans *et al*., 2015). Despite being generally vulnerable to effects from varying phycobilisome content and state transitions, both of these metrics ($\Phi_{PSII}$ and qP) remain reliable to elucidate reduced photosynthetic efficiency as they directly correlate with oxygen evolution rates within an identical strain over short time scales, such as the conditions utilized in this study (Ogawa *et al*., 2013; Osanai *et al*., 2013;

Misumi *et al*., 2016). Alteration of photosynthetic parameters in response to abiotic stress was not a result of carbon limitation, as supplementation with $HCO_3^-$ did not significantly affect the results. Here we demonstrate that both $\Phi_{PSII}$ and qP decrease in response to all tested stresses, an effect that directly correlates with increased CrhR abundance. Since these experiments were performed in the presence of constant light, they suggest a more closed PSII, indicative of enhanced reduction of the ETC downstream of $Q_A$ and the potential involvement of $PQH_2$. In agreement with our conclusions, independent PAM measurements have also shown that PQ becomes reduced upon temperature downshift of *Synechocystis* cells, as a consequence of enhanced respiratory electron flow into the PQ pool (Sireesha *et al*., 2012) and with decreased photosynthetic consumption of $NADPH_2$ (Rosana *et al*., 2012b) contributing to further reduction of the pool. Similarly, analysis of $Q_A$ re-oxidation kinetics demonstrated that $Q_A$ reduction progressively becomes more elevated following a saturating pulse of light as temperature decreased. A similar linear response of fluorescence and the correlated changes in CrhR abundance were also observed for four additional stresses. These responses suggest that there is a linear response, beyond a certain threshold, between $PQH_2$:PQ and CrhR accumulation. This stress induced over-reduction of PQ is accompanied by a significant increase in non-regulated energy dissipation ($\Phi_{f,\,D}$), suggesting enhanced reduction of the ETC under abiotic stress conditions causes overall photosynthetic efficiency to decrease. This is not the case for regulated energy dissipation ($\Phi_{NPQ}$), as changes in $\Phi_{NPQ}$ were not correlated with stresses shown to induce CrhR.

### 2.5.4 *Convergent sensing and two-component signal transduction*

Previous work has indicated that a combination of ETC inhibitors and PAM fluorometry have been used to show that $PQH_2$ regulates expression of a diverse set of genes in cyanobacteria (Kujat and Owttrim, 2000; El Bissati and Kirilovsky, 2001; Ivleva *et al*., 2006; Kim *et al*., 2012; Sireesha *et al*., 2012). The data presented here indicate that CrhR expression is regulated by a diverse range of abiotic stresses that are convergently sensed by their common effect on the redox poise of $Q_B$ and/or $PQH_2$. Since bacteria typically sense abiotic stress through independent histidine kinase sensors associated with two-component signal transduction pathways (Capra and Laub, 2012), our data detailing

sensing of multiple stresses via a common sensor, the redox status of $Q_B/PQH_2$, is potentially unexpected. While convergent signaling pathways frequently occur, convergent sensing pathways are less common (Shen and Fang, 2012). However, our proposed convergent sensing system would require a downstream, traditional two-component signal transduction pathway to sense and transmit the stress alteration of the redox poise of $Q_B/PQH_2$ to regulate *crhR* expression (Fig. 2.1). While the exact nature of the two-component system functioning in CrhR regulation is not known, a similar system in *Arabidopsis thaliana* may provide insights into the nature of such a pathway. In this system, a similar ETC dependent sensing system appears to occur in which the protein kinases KIN10/11 control reprogramming of transcription in response to darkness, sugar and developmental signals to globally regulate plant metabolism, energy balance and growth, pathway effects mimicked by *crhR* mutation (Baena-González *et al*., 2007; Rosana *et al*., 2012b). The involvement of ETC-mediated redox regulation is indicated by the DCMU-induced accumulation of KIN10/11. A potential upstream effector of KIN10/11 is the sensor kinase CSK that regulates *psaA* expression through direct binding to quinones in chloroplasts (Puthiyaveetil *et al*., 2008). In contrast to the CSK/KIN system, we envision that the two-component signal transduction chain downstream of convergent redox sensing activates CrhR expression. CrhR can then function as a post-translational effector rather than as a transcription factor. In conjunction, it has recently been shown that deletion of *crhR* causes decreased expression of the flavodiiron proteins *flv4* and *flv3*, as well as *menB* ncRNA, which participate in photoprotection at PSII and PSI and quinone metabolism respectively, suggesting *crhR* may be involved in the rapid acclimation of the ETC to diverse abiotic stresses (Georg *et al*., 2019).

The proposal that CSK evolved from the essential cyanobacteria histidine kinase Hik2 (Puthiyaveetil *et al*., 2008) implicates Hik2 as a potential sensor of PQ redox status in cyanobacteria, however this remains to be proven (Ibrahim *et al*., 2016). In addition, it should be noted that while Hik2 has been reported to be associated with sensing of multiple stresses in cyanobacteria, evidence suggests that Hik2 responds primarily to chloride ion concentration (Kotajima *et al*., 2014) and does not possess transmembrane domains. Thus, the mechanisms by which Hik2 performs redox sensing and elicits signal transduction associated with PQ are not known (Ibrahim *et al*., 2016). Whatever the Hik sensor, it

appears that the response regulators Rre33 and Rre1 may perform roles in this signal transduction pathway (Li and Sherman, 2000; Ibrahim *et al*., 2016) (Fig. 2.1). Thus, if Hik2 dependent signaling controls *crhR* expression downstream of PQ, the convergent sensing mechanism described here may be conserved across photosynthetic organisms. In addition, the response of gene expression to nitric oxide and hypoxia stress in *Staphylococcus aureus* is controlled by the SrrAB two-component system. This system requires functional quinol, again suggesting a role for the ETC redox poise in stress perception (Kinkel *et al*., 2013). These two examples provide provocative evidence for gene regulation via a common effect of abiotic stress on the redox poise of the ETC and suggest convergent sensing systems maybe more conserved than anticipated.

In summary, a variety of techniques provided evidence to support the hypothesis that a convergent sensing mechanism detects a range of abiotic stress via their common effect on the redox poise of the ETC in *Synechocystis* most likely within $Q_B$/$PQH_2$. The redox poise then functions as a redox switch to regulate gene expression. Here, increased reduction of $Q_B$/$PQH_2$ would be sensed by a two-component signal transduction pathway to regulate downstream gene expression, including enhanced CrhR RNA helicase abundance (Fig. 2.1). The sensor kinase Hik2 and the response regulators Rre33 and Rre1 are likely candidates that may perform roles in this signal transduction pathway (Li and Sherman, 2000; Ibrahim *et al*., 2016). We propose that CrhR constitutes a significant effector of the general stress response downstream of this signaling cascade. The ability of the induced RNA helicase to catalyze duplex RNA unwinding and annealing (Chamot *et al*., 2005) provides a mechanism by which expression of a diverse set of genes required for stress acclimation could be regulated at the post-transcriptional level. This regulatory system would be anticipated to be intimately associated with crucial aspects of cellular homeostasis including the circadian clock, cell growth and metabolism. Overall, the convergent sensing mechanism would be integrated into a regulatory network providing an efficient and rapid response to a variety of abiotic stresses. The interplay and possible crosstalk between these pathways require further analysis.

## 2.6 References


Baena-González, E., Rolland, F., Thevelein, J.M. and Sheen, J. 2007. A central integrator of transcription networks in plant stress and energy signalling. Nature 448, 938–942.

Beier D, Gross R. 2006. Regulation of bacterial virulence by two-component systems. Current Opinion in Microbiology 9, 143–152.

Berla BM, Saha R, Maranas CD, Pakrasi HB. 2015. Cyanobacterial alkanes modulate photosynthetic cyclic electron flow to assist growth under cold stress. Scientific Reports 5, 14894.

Belatik A, Joly D, Hotchandani S, Carpentier R. 2013. Re-evaluation of the side effects of cytochrome $b_6f$ inhibitor dibromothymoquinone on photosystem II excitation and electron transfer. Photosynthesis Research 117, 489–496.

Bode R, Ivanov AG, Hüner NP. 2016. Global transcriptome analyses provide evidence that chloroplast redox state contributes to intracellular as well as long-distance signalling in response to stress and acclimation in *Arabidopsis*. Photosynthesis Research 128, 287–312.

Calzadilla PI, Zhan J, Sétif P, Lemaire C, Solymosi D, Battchikova N, Wang Q, Kirilovsky D. 2019. The cytochrome $b_6f$ complex is not involved in cyanobacterial state transitions. Plant Cell 31, 911–931.

Campbell D, Hurry V, Clarke AK, Gustafsson P, Oquist G. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiology and Molecular Biology Reviews 62, 667–683.

Capra EJ, Laub MT. 2012. Evolution of two-component signal transduction systems. Annual Review of Microbiology 66, 325–347.

Chamot D, Colvin KR, Kujat-Choy SL, Owttrim GW. 2005. RNA structural rearrangement via unwinding and annealing by the cyanobacterial RNA helicase, CrhR. Journal of Biological Chemistry 280, 2036–2044.

Derks A, Schaven K, Bruce D. 2015. Diverse mechanisms for photoprotection in photosynthesis. Dynamic regulation of photosystem II excitation in response to rapid environmental change. Biochimica et Biophysica Acta 1847, 468–485.

Dietz KJ. 2015. Efficient high light acclimation involves rapid processes at multiple mechanistic levels. Journal of Experimental Botany 66, 2401–2414.

Dietz KJ, Pfannschmidt T. 2011. Novel regulators in photosynthetic redox control of plant metabolism and gene expression. Plant Physiology 155, 1477–1485.

Eaton SL, Roche SL, Llavero Hurtado M, Oldknow KJ, Farquharson C, Gillingwater TH, Wishart TM. 2013. Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. PLoS ONE 8, e72457.

El Bissati K, Kirilovsky D. 2001. Regulation of *psbA* and *psaE* expression by light quality in *Synechocystis* species PCC 6803. A redox control mechanism. Plant Physiology 125, 1988–2000.

Fan DY, Jia H, Barber J, Chow WS. 2009. Novel effects of methyl viologen on photosystem II function in spinach leaves. European Biophysics Journal 39, 191–199.

Georg J, Rosana ARR, Chamot D, Migur A, Hess WR, Owttrim GW. 2019. Inactivation of the RNA helicase CrhR impacts a specific subset of the transcriptome in the cyanobacterium *Synechocystis* sp. PCC 6803. RNA Biology 16, 1205–1214.

Genty B, Briantais J-M, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta 990, 87–92.

Hakkila K, Antal T, Rehman AU, Kurkela J, Wada H, Vass I, Tyystjärvi E, Tyystjärvi T. 2014. Oxidative stress and photoinhibition can be separated in the cyanobacterium *Synechocystis* sp. PCC 6803. Biochimica et Biophysica Acta 1837, 217–225.

Ibrahim IM, Puthiyaveetil S, Allen JF. 2016. A two-component regulatory system in transcriptional control of photosystem stoichiometry: Redox-dependent and sodium ion-dependent phosphoryl transfer from cyanobacterial histidine kinase Hik2 to response regulators Rre1 and RppA. Frontiers in Plant Science 7, 137.

Ivleva NB, Gao T, LiWang AC, Golden SS. 2006. Quinone sensing by the circadian input kinase of the cyanobacterial circadian clock. Proceedings of the National Academy of Sciences of the United States of America USA 103, 17468–17473.

Jung K, Fried L, Behr S, Heermann R. 2012. Histidine kinases and response regulators in networks. Current Opinion in Microbiology 15, 118–124.

Kanesaki Y, Yamamoto H, Paithoonrangsarid K, Shoumskaya M, Suzuki I, Hayashi H, Murata N. 2007. Histidine kinases play important roles in the perception and signal transduction of hydrogen peroxide in the cyanobacterium, *Synechocystis* sp. PCC 6803. Plant Journal 49, 313–324.

Kim YI, Vinyard DJ, Ananyev GM, Dismukes GC, Golden SS. 2012. Oxidized quinones signal onset of darkness directly to the cyanobacterial circadian oscillator. Proceedings of the National Academy of Sciences of the United States of America USA 109, 17765–17769.

Kinkel TL, Roux CM, Dunman PM, Fang FC. 2013. The *Staphylococcus aureus* SrrAB two-component system promotes resistance to nitrosative stress and hypoxia. MBio 4, e00696–13.

Kotajima T, Shiraiwa Y, Suzuki I. 2014. Functional analysis of the N-terminal region of an essential histidine kinase, Hik2, in the cyanobacterium *Synechocystis* sp. PCC 6803. FEMS Microbiology Letters 351, 88–94.

Kujat SL, Owttrim GW. 2000. Redox-regulated RNA helicase expression. Plant Physiology 124, 703–714.

Li H, Sherman LA. 2000. A redox-responsive regulator of photosynthesis gene expression in the cyanobacterium *Synechocystis* sp. Strain PCC 6803. Journal of Bacteriology 182, 4268–4277.

Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N. 2003. Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis* sp. PCC 6803. Proceedings of the National Academy of Sciences of the United States of America USA 100, 9061–9066.

Mikami K, Kanesaki Y, Suzuki I, Murata N. 2002. The histidine kinase Hik33 perceives osmotic stress and cold stress in *Synechocystis* sp PCC 6803. Molecular Microbiology 46, 905–915.

Misumi M, Katoh H, Tomo T, Sonoike K. 2016. Relationship Between Photochemical Quenching and Non-Photochemical Quenching in Six Species of Cyanobacteria Reveals Species Difference in Redox State and Species Commonality in Energy Dissipation. Plant and Cell Physiology 57, 1510–1517.

Mullineaux CW. 2013. Co-existence of photosynthetic and respiratory activities in cyanobacterial thylakoid membranes. Biochimica et Biophysica Acta 1837, 503–511.

Mullineaux CW, Allen JF. 1986. The state 2 transition in the cyanobacterium *Synechococcus* 6301 can be driven by respiratory electron flow into the plastoquinone pool. Federation of European Biochemical Societies Letters 205, 155–160.

Mullineaux CW, Allen JF. 1990. State 1-State 2 transitions in the cyanobacterium *Synechococcus* 6301 are controlled by the redox state of electron carriers between Photosystems I and II. Photosynthesis Research 23, 297–311.

Murata N, Suzuki I. 2006. Exploitation of genomic sequences in a systematic analysis to access how cyanobacteria sense environmental stress. Journal of Experimental Botany 57, 235–247.

Nishiyama Y, Yamamoto H, Allakhverdiev S, Inaba M, Yokota A, Murata N. 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. EMBO Journal 20, 5587–5594.

Ogawa T, Harada T, Ozaki H, Sonoike K. 2013. Disruption of the *ndhF1* gene affects Chl fluorescence through state transition in the Cyanobacterium *Synechocystis* sp. PCC 6803, resulting in apparent high efficiency of photosynthesis. Plant And Cell Physiology 54, 1164–1171.

Ogawa T, Misumi M, Sonoike K. 2017. Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions. Photosynthesis Research 133, 63–73.

Osanai T, Kuwahara A, Iijima H, Toyooka K, Sato M, Tanaka K, Ikeuchi M, Saito K, Hirai MY. 2013. Pleiotropic effect of *sigE* over-expression on cell morphology, photosynthesis and hydrogen production in *Synechocystis* sp. PCC 6803. Plant Journal 76, 456–465.

Owttrim GW. 2012. RNA helicases in cyanobacteria: biochemical and molecular approaches. Methods in Enzymology 511, 385–403.

Owttrim GW. 2013. RNA helicases: diverse roles in prokaryotic response to abiotic stress. RNA Biology 10, 96–110.

Oxborough K, Baker NR. 1997. Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of qP and Fv-/Fm-; without measuring Fo-. Photosynthesis Research 54, 135–142.

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I, et al. 2004. Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. Journal of Biological Chemistry 279, 53078–53086.

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF. 2008. The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. Proceedings of the National Academy of Sciences of the United States of America USA 105, 10061–10066.

Rosana AR, Chamot D, Owttrim GW. 2012a. Autoregulation of RNA helicase expression in response to temperature stress in *Synechocystis* sp. PCC 6803. PLoS ONE 7, e48683.

Rosana AR, Ventakesh M, Chamot D, Patterson-Fortin LM, Tarassova O, Espie GS, Owttrim GW. 2012b. Inactivation of a low temperature-induced RNA helicase in *Synechocystis* sp. PCC 6803: physiological and morphological consequences. Plant and Cell Physiology 53, 646–658.

Rastogi RP, Singh SP, Häder DP, Sinha RP. 2010. Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate in the

cyanobacterium *Anabaena variabilis* PCC 7937. Biochemical and Biophysical Research Communications 397, 603–607.

Rowland MA, Deeds EJ. 2014. Crosstalk and the evolution of specificity in two-component signaling. Proceedings of the National Academy of Sciences of the United States of America USA 111, 5550–5555.

Schneider CA, Rasband WS, Eliceiri KW. 2012. "NIH Image to ImageJ: 25 years of image analysis", Nature Methods 9, 671–675.

Schreiber U. 1986. Detection of rapid induction kinetics with a new type of high frequency modulated chlorophyll fluorometer. Photosynthesis Research 9, 261–272.

Schuurmans RM, van Alphen P, Schuurmans JM, Matthijs HC, Hellingwerf KJ. 2015. Comparison of the Photosynthetic Yield of Cyanobacteria and Green Algae: Different Methods Give Different Answers. PLoS ONE 10, e0139061.

Schuurmans RM, Matthijs JC, Hellingwerf KJ. 2017. Transition from exponential to linear photoautotrophic growth changes the physiology of *Synechocystis* sp. PCC 6803. Photosynthesis Research 132, 69–82.

Sétif P. 2015. Electron-transfer kinetics in cyanobacterial cells: methyl viologen is a poor inhibitor of linear electron flow. Biochimica et Biophysica Acta 1847, 212–222.
Shen S, Fang FC. 2012. Integrated stress responses in *Salmonella*. International Journal of Food Microbiology 152, 75–78.

Sireesha K, Radharani B, Krishna PS, Sreedhar N, Subramanyam R, Mohanty P, Prakash JS. 2012. RNA helicase, CrhR is indispensable for the energy redistribution and the regulation of photosystem stoichiometry at low temperature in *Synechocystis* sp. PCC6803. Biochimica et Biophysica Acta 1817, 1525–1536.

Slabas AR, Suzuki I, Murata N, Simon WJ, Hall JJ. 2006. Proteomic analysis of the heat shock response in *Synechocystis* PCC6803 and a thermally tolerant knockout strain lacking the histidine kinase 34 gene. Proteomics 6, 845–864.

Suzuki I, Kanesaki Y, Mikami K, Kanehisa M, Murata N. 2001. Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. Molecular Microbiology 40, 235–244.
Suzuki I, Kanesaki Y, Hayashi H, Hall JJ, Simon WJ, Slabas AR, Murata N. 2005. The histidine kinase Hik34 is involved in thermotolerance by regulating the expression of heat shock genes in *Synechocystis*. Plant Physiology 138, 1409–1421.

Tarassova OS, Chamot D, Owttrim GW. 2014. Conditional, temperature-induced proteolytic regulation of cyanobacterial RNA helicase expression. Journal of Bacteriology 196, 1560–1568.

Trebst A. 1980. Inhibitors in electron flow: tools for the functional and structural localization of carriers and energy conservation sites. Methods in Enzymology 69, 675–715.

Trebitsh T, Danon A. 2001. Translation of chloroplast *psbA* mRNA is regulated by signals initiated by both photosystems II and I. Proceedings of the National Academy of Sciences of the United States of America USA 98, 12289–12294.

Tu CJ, Shrager J, Burnap RL, Postier BL, Grossman AR. 2004. Consequences of a deletion in *dspA* on transcript accumulation in *Synechocystis* sp. strain PCC6803. Journal of Bacteriology 186, 3889–3902.

Vinnemeier J, Hagemann M. 1999. Identification of salt-regulated genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803 by subtractive RNA hybridization. Archives of Microbiology 172, 377–386.

Wardman P. 2007. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls and prospects. Free Radical Biology and Medicine 43, 995–1022.

Wilde A, Hihara Y. 2016. Transcriptional and posttranscriptional regulation of cyanobacterial photosynthesis. Biochimica et Biophysica Acta 1857, 296–308.

# Chapter 3: Distinct light signals regulate *crhR* RNA helicase expression in *Synechocystis* sp. PCC 6803

## 3.1 Abstract

Photosynthetic organisms must be able to sense and respond to light conditions for survival. Similar to plants and algae, cyanobacteria have evolved a range of mechanisms to sense and respond to the fluctuating light conditions experienced in their natural environment. We demonstrate here that cyanobacteria integrate a series of three distinct light signals to generate a complex network regulating expression of the DEAD-box RNA helicase, *crhR*, at discrete points. An interlinked experimental system designed to assess the contribution of light, redox, oxygen and ROS on CrhR abundance combined with ETC inhibitors indicated that CrhR protein accumulation was regulated by light-driven alteration of the redox poise of PQ. Exposure to different wavelengths of light revealed a light-quality effect where a red-light signal is required for *crhR* translation. Finally, examination of repression of CrhR expression in the experimental conditions described above indicated that a redox- and light quality-independent signal was required for CrhR degradation. The crucial role of light was further revealed by the observation that dark conditions superseded the light signals required to initiate each of these processes. The findings provide new insights, revealing an unexpected complexity of light sensing and signaling associated with expression of a single gene in cyanobacteria.

## 3.2 Introduction

Light is crucial for the survival of photosynthetic organisms, especially cyanobacteria. Many cyanobacteria are obligate photoautotrophs, and thus obtain all of their energy from light harvesting and carbon skeletons from photosynthetic carbon fixation. In order to survive it is therefore imperative that cyanobacteria sense and rapidly respond to the fluctuating light environment they constantly encounter in their natural habitat. Cyanobacteria and higher plants therefore possess a range of light sensing systems that regulate gene expression and control a diverse range of physiological and metabolic processes (Montgomery, 2007; Rockwell and Lagarias, 2017; Fujisawa and Masuda, 2018; Wiltbank and Kehoe, 2019).

In all photosynthetic organisms, light driven alteration of the redox potential of the photosynthetic electron transport chain (ETC) is a known sensor that regulates gene expression. Signal transduction pathways originating from the redox poise of the ETC components plastoquinone (PQ) and ferredoxin (Fd) regulate gene expression in cyanobacteria, algae and higher plants (Ibrahim et al., 2016; Locato et al., 2018) and are crucial to integrating energy homeostasis with core metabolism (Westermark and Steuer, 2016) and response to abiotic stress (Kujat and Owttrim, 2000; Ritter et al., 2019). Since ROS production is oxygen dependent and a natural consequence of light driven electron flow through the ETC in photosynthetic organisms, identification of gene expression regulated by each of these signals is crucial. Barth et al. (2014) addressed the light-redox-oxygen-ROS regulation problem by designing an interlinked experimental system to assess the contribution of each stress to protein abundance. The authors used a proteomics approach to determine protein abundance changes in response to all four possible combinations of light intensity (high light (HL) and low light (LL)) and oxygen concentration (aerobic vs microaerobic) in *Chlamydomonas reinhardtii*. Five regulatory mechanisms were identified, Light, Redox II, ROS, Light-repressed and 4-Down, that regulated different protein expression profiles in *Chlamydomonas* (Barth et al., 2014).

Light quality also facilitates redox-independent regulation typically utilizing protein bound chromophores as photoreceptors that are activated by specific wavelengths of light (Balasubramanian et al., 2006). Since light detection is essential for survival, the photoreceptor complement encoded in cyanobacteria is expanded; *Nostoc punctiforme*

encodes 21 different phytochrome superfamily proteins that sense light across the visible spectrum (Campbell et al., 2015). While phytochrome domain structures in higher plants are relatively invariant, those found in prokaryotes are more variable (Rockwell et al., 2017). One unique property is the tendency for cyanobacteria to express multiple photosensory modules with varying spectroscopic properties associated with a single protein, allowing integration of information from multiple regions of the light spectrum allowing adaptation of photosynthetic light-harvesting gene expression to varying light quality (Wiltbank and Kehoe, 2016; Wiltbank and Kehoe, 2019). Red light in particular appears to be perceived uniquely by cyanobacteria, due to light harvesting occurring primarily through phycobilisomes that have negligible absorption at wavelengths below 495 nm (Grossman et al., 1993). As a result, it has been recently shown that while blue light (PSI) is absorbed to a similar extent as red light (PSII), it is much less effectively utilized for oxygenic photosynthesis and growth in *Synechocystis* PCC 6803 (hereafter *Synechocystis*), a very different situation than observed in algae and higher plants (Luimstra et al., 2018). Blue light perception and response is responsible for regulation of various physiological processes in *Synechocystis*, including flocculation (Conradi et al., 2019) and phototaxis (Fujisawa and Masuda, 2018) via activation of photoreceptor Cph2 production of c-di-GMP, is established. In addition, short daily exposures of blue light are essential for mixotrophic growth, suggesting that while not important as a source of energy, blue light plays an important role as an environmental signal regulating cyanobacterial metabolism (Anderson and McIntosh, 1991).

Phytochrome is known to regulate gene expression via diverse mechanisms including light quality-dependent regulation of translation. For example, in *Arabidopsis thaliana* the light activated form of phytochrome (Pfr) interacts with PENTA1 (Pnt1) bound to the 5' UTR of the protochlorophyllide reductase (PORA) transcript, to inhibit translation through an unknown mechanism under non-permissive conditions (Paik et al., 2012). Repression is relieved by conversion of phytochrome to the Pr form in the dark, allowing PORA translation. A similar system may operate in *Synechocystis* where synthesis of the D1 protein encoded by the *psbA* mRNA is regulated at the level of translation elongation (Tyystjärvi et al., 2001). For *psbA*, translation initiation is not regulated, instead, elongation is arrested in the dark via an unknown mechanism. An

unspecified light signal then targets the ribosome-PsbA nascent chain complex to the thylakoid membrane where translation is completed. Another aspect of light quality regulation that has been extensively studied in cyanobacteria is chromatic acclimation (CA), the process by which cyanobacteria adapt phycobilisome composition to optimize light absorption (Kehoe, 2010.) CA regulatory pathways primarily regulate transcript accumulation using phytochrome superfamily members containing histidine kinase domains (Wiltbank and Kehoe, 2019). However, regulation of one CA pathway, control of the green-light induction pathway (Cgi), operates post- transcriptionally by transcription attenuation to control phycoerythrin synthesis in *Fremyella diplosiphon*. This mechanism involves regulation of translation initiation where translation initiation factor 3 (IF3) is proposed to affect transcriptional-translational coupling, enhancing transcription attenuation in red light, the non-permissive condition, but allowing expression in green light (Bezy et al., 2011; Gutu et al., 2013). Thus, expression of genes associated with crucial aspects of photosynthesis and light harvesting are regulated by a variety of light signals. However, regulation of a single gene by multiple light signals has, as far as we are aware, not been reported.

In addition, light quality and quantity and the relative redox poise of the ETC differentially regulate distinct aspects of the photosynthetic machinery. For example, light quantity drives state transition, the reorganization of light harvesting proteins associated with the photosynthetic reaction centers, facilitating the reallocation of light energy between the photosystems (Joshua and Mullineaux, 2004; Mullineaux and Emlyn-Jones, 2005). While in higher plants and algae, state transition is controlled by the redox poise of cytochrome $b_6f$ (cyt $b_6f$) and depends on a phosphorylation cascade to enact changes in gene expression, this is not the case in cyanobacteria, suggesting signal transduction of ETC derived signals differs between the two groups (Calzadilla et al., 2019). In contrast, changes in PSI coding gene expression responds to the ETC inhibitors DCMU and DBMIB and PSI regulation can be rescued by enhanced respiratory electron flow into the PQ pool by metabolism of exogenous glucose, indicative of redox regulation (Mullineux and Allen, 1986; Mullineaux and Allen, 1990; Alfonso et al., 2000). Glucose rescue results from the respiratory and photosynthetic ETC sharing components in *Synechocystis* (Hirano et al.,

1980). These examples indicate there is a distinction between light and redox responsive gene regulatory mechanisms.

Expression of *crhR*, encoding the single DEAD box RNA helicase in the photosynthetic, oxygen evolving model cyanobacterial species, *Synechocystis*, is regulated by a range of abiotic stresses via their common effect on the redox potential of PQ in the ETC (Kujat and Owttrim, 2000; Ritter et al., 2019). RNA helicases function in all aspects of RNA metabolism through the ATP-dependent rearrangement of RNA secondary structure or ribonucleoprotein interactions (Linder and Jankowsky, 2011). These RNA secondary structure alterations are known to regulate gene expression, frequently in response to abiotic stress and in association with alteration of small-regulatory RNA (sRNA) activity (Linder and Owttrim, 2009; Linder and Jankowski, 2011; Owttrim, 2013). Associated with the redox poise of PQ, temperature shift dramatically affects *crhR* expression, downshift from 30ºC to 20ºC enhances both transcript and protein abundance by 10-15 fold (Rosana et al., 2012a; Ritter et al., 2019) while subsequent upshift to 30ºC induces temperature-dependent, conditional proteolysis of CrhR (Tarassova et al., 2014). These temperature-mediated regulatory pathways involve a complex interconnected network consisting of a number of CrhR-dependent auto-regulatory and CrhR-independent steps (Rosana et al., 2012a). Thus, evidence has been provided indicating that the light-driven alteration of the PQ redox poise regulates *crhR* expression, coupling gene expression with cellular energy homeostasis and survival at 20ºC (Kujat and Owttrim, 2000; Rosana et al., 2012b; Ritter et al., 2019).

At low temperature, *crhR* inactivation causes photosynthetic carbon fixation to cease which, combined with alterations in gene expression, lead to severe morphological alterations and a severe cold-sensitive phenotype (Rosana et al., 2012b). Induced CrhR generates these effects by influencing all stages of RNA metabolism, including transcription, translation and turnover (Georg et al., 2019), presumably through CrhR catalyzed rearrangement of RNA secondary structure via duplex unwinding and annealing (Chamot et al., 2005). Recently, we have shown that CrhR is a major regulator of both messenger and non-coding RNAs in cold- stressed *Synechocystis,* with the flavodiiron genes *flv4*/3/2, as well as PmgR1, an sRNA involved in photomixotrophic growth, differentially expressed in $crhR_{TR}$ cells (Georg et al., 2019). In addition, CrhR is induced

by a range of abiotic stresses independent of low temperature stress, by their common effect on the redox poise of PQ (Ritter et al., 2019). Combined, these studies suggested that CrhR is required for adaptation of photosynthetic gene expression in response to varying environmental conditions that potentially could cause over-reduction of the PQ pool. Interestingly, divergent results have been presented regarding the requirement of light for low temperature induction of *crhR* transcript accumulation. We initially reported that *crhR* was not detected in dark incubated cells at 20ºC but rapidly accumulates in the presence of light (Kujat and Owttrim, 2000). Subsequently, data was presented indicating that light was not required for *crhR* transcript accumulation at 20ºC, as transcript was detected in cold treated cells in the dark (Mironov et al., 2012; Mironov et al., 2014). At the protein level, evidence has been presented indicating that light was required for CrhR accumulation (Kujat and Owttrim, 2000; Chen et al., 2018). Overall, these results suggested that light potentially performs roles at multiple levels associated with *crhR* expression.

Here we expand this analysis by further investigating the contribution of light to *crhR* expression. The results reveal that three discrete light signals facilitate regulation at multiple stages of gene regulation, from transcript accumulation to protein degradation. We demonstrate that *crhR* expression requires light signals associated with: 1) light-driven alteration of the redox poise of PQ, required for transcript accumulation in response to abiotic stress; 2) light quality where CrhR translation, but not transcript accumulation, specifically requires red light; and 3) a light signal *per se*, independent of redox poise and wavelength, required for the proteolytic repression of CrhR abundance in the absence of stress.

## 3.3 Materials and Methods

### *3.3.1 Strains and culture conditions*

The glucose tolerant strain of *Synechocystis* sp. PCC 6803 (Kujat and Owttrim, 2000) was grown under standard conditions in BG-11 medium at 30°C under continuous white light and aerated by shaking at 150 rpm and bubbling with humidified air as described previously (Owttrim, 2012). When described, a genomic truncation mutant of the *crhR* ORF, *crhR*$_{TR}$, was grown in the presence of spectinomycin and streptomycin, both at 50 mg/mL (Rosana, 2012b). The *crhR*$_{TR}$ strain produces a truncated version of the *crhR*

transcript and CrhR polypeptide that is biochemical inactive (Rosana et al,, 2012). Bubbling with exogenous gasses was performed as described for humidified air. A microaerobic environment was provided by bubbling with a 95% $N_2$ – 5% $CO_2$ mixture. Photosynthetically active radiation (36 μmol $m^{-2}$ $s^{-1}$, 400 – 700 nm) was provided by a bank of warm-white fluorescent lamps and corresponded to low light (LL) conditions. For high light conditions (HL), warm-white fluorescent lamps provided photosynthetically active radiation at 150 $m^{-2}$ $s^{-1}$, 400 – 700 nm. Light quality was altered using Innova Dual Spectrum LED Grow Lights providing red (662 nm) or blue (455 nm) illumination at 170 mW/nm or 160 mW/nm, respectively. Experiments utilized cultures with chlorophyll *a* (Chl *a*) concentrations ranging from 5-7 μg/mL.

*3.3.2 Stress and inhibitor conditions*

Unless otherwise stated, illuminated mid-log phase cultures were subjected to the following stress conditions for the indicated times: 20°C cold stress, high light (HL) (140 μmol photons $m^{-2}$ $s^{-1}$), glucose 5 mM. Enhanced $CO_2$ levels were provided by bubbling with 5% $CO_2$ in either nitrogen or air to provide either microaerobic (maer) or aerobic conditions (aer), respectively. The electron transport inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 2.5 μM) and dibromothymoquinone (DBMIB, 10 μM) were dissolved in DMSO as described previously (Kujat and Owttrim, 2000). DMSO (0.066%) was added to a control culture when ETC inhibitors were used.

*3.3.3 Protein abundance*

Wild type CrhR (55 kDa) abundance was determined by Western blot analysis of soluble protein (20 ug) using anti-CrhR antibody (1:5,000) (Kujat and Owttrim, 2000) and ECL detection (ECL, Bio-Rad Clarity Western ECL Substrate, Mississauga, ON), as previously described (Owttrim, 2012). When shown, the abundance of Rps1 was used as a protein loading control using anti-*Escherichia coli* Rps1 (1:5,000) and ECL detection, as previously described (Rosana et al., 2012a). Soluble protein was quantified using a Bradford assay with BSA as the standard.

*3.3.4 RNA sampling and extraction*

Cultures were harvested at the growth temperature either by vacuum filtration or by directly adding an aliquot to an equal volume of 5% phenol in EtOH. Cells in 5% phenol in EtOH were pelleted by centrifugation at 4ºC for 15 min (6,000*g*). Cell pellets were either frozen at -80ºC for up to three days or used immediately for RNA extraction. Cells were washed once with 50:100 TE and pellets resuspended in lysis buffer (0.5% Triton X100, 0.5% N-lauroyl sarcosine, and 0.4% SDS in 50:100 TE). RNA was extracted via glass bead lysis in the presence of phenol followed by phenol-chloroform extraction and overnight precipitation with 4 M lithium chloride as described in Owttrim (2012). Following precipitation from lithium chloride, RNA was resuspended in sterile water and precipitated at -80ºC with 0.10 volume NaOAc (3M, pH 5.2) and 2.5 volume ethanol (100%).

*3.3.5 Quantification of RNA and quality confirmation*

RNA concentration was determined using a NanoDrop spectrophotometer (ThermoScientific) and RNA quantity and quality was visualized by electrophoresis of denatured RNA (5 µg) on a MOPS-buffered 1.2% formaldehyde-agarose gel (Owttrim, 2012). RNA quality was further confirmed using an Agilent 2100 Bioanalyzer, as per the manufacturer's protocol. A RIN number >8.0 was required before proceeding with cDNA generation.

*3.3.6 cDNA generation*

Genomic DNA was removed from RNA samples (0.2 µg/µl) by treatment twice with DNase I (0.04 U/µl, Ambion) for 30 min at 37ºC. Use of DNase I treated RNA for standard PCR did not produce detectable amplification. cDNA was generated using DNase I treated RNA, random hexamers (Invitrogen), and dNTPS (Fermentas) at 0.17 µg/µl, 50 ng/µl and 0.5 mM respectively. This mixture was hybridized in a Mastercycler Personal Thermal Cycler 5331 (Eppendorf) to 70ºC for 10 min, followed by 25ºC for 10 min, diluted in 5X First Strand Buffer (Invitrogen) before addition of DTT (10 mM, Invitrogen) and Superscript II (5 U/µl, Invitrogen). cDNA generation was performed using thermocycling at 25ºC for 10 min, 37ºC for 60 min, 42ºC for 60 min, 70ºC for 10 min.

*3.3.7 Primer design and selection of endogenous control gene*

Three endogenous controls previously identified from microarray data were tested (Pinto et al. (2012). Primer pairs for *rnpB*, *rrn16sb* and *petB* were designed using Primer ExpressTM 3.0.1 software (Thermo-Fisher Scientific) (Table 1). All three control genes gave constant abundance over various growth conditions and *rnpB* was selected as the endogenous control for ΔΔCT analysis. Primer annealing efficiency was determined to be >98% before further use.

*3.3.8 qPCR amplification*

qPCR reactions were performed in triplicate in 96 well plates (MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, Applied Biosystems). Each well contained 5 µl of 2X SYBR Mastermix (MBSU, University of Alberta), gene-specific forward and reverse primers (3.2 µM) and 2.5 µl diluted cDNA template (1:1024) in a final reaction volume of 10 µl. Amplification was performed by thermocycling at 95ºC for 2 min followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min on a 7500 Fast Real-Time PCR system (Applied Biosystems) at standard ramp speed. Melt curves were generated at 1ºC intervals from 60ºC to 95ºC.

*3.3.9 Data manipulation*

Transcript abundance was normalized to *rnpB* and reported as ΔΔCT. Significance was determined using a Student's T-Test ($p>0.05$). Raw data was collected from the "7500 Software V2.3" (Applied Biosystems) before statistical analysis and graphing performed in the" GraphPad Prism 8 software version 8.0.1" (GraphPad Software, Inc., La Jolla, CA, USA).

## 3.4 Results

We have recently established that CrhR expression is controlled by a convergent sensing mechanism, activated by the common effect of abiotic stresses enhancing the redox poise of PQ in the ETC (Ritter et al., 2019). In order to differentiate redox expression from similar responsive mechanisms that regulate gene expression in photosynthetic organisms,

**Table 3.1 Primers tested for qPCR analysis**

| | |
|---|---|
| *crhR* Forward Primer | GATCGCCGCTGCTGCTT |
| *crhR* Reverse Primer | GGCACTTCCCAATCGGATT |
| *rnpB* Forward Primer | TGTCACAGGGAATCTGAGGAAAGT |
| *rnpB* Reverse Primer | CTGTTTACTGGTTGCTGTTTTCTAAAA |
| *petB* Forward Primer | TGGGCGGTAAAAATCGTTTC |
| *petB* Reverse Primer | TCGCATGAGGGTCACCAATT |
| *rrn16sb* Forward Primer | ATCAAACCCGGCCTCAGTTC |
| *rrn16sb* Reverse Primer | ACCTGCGATTACTAGCGATTCC |

experiments combining high light (HL) or low light (LL) under aerobic or microaerobic conditions were performed. The experimental design utilizes all combinations of low and high light and oxygen levels to generate eight growth conditions, as summarized in Figure 3.1A. As shown in Figure 3.1B, under aerobic conditions CrhR abundance was induced by HL and subsequently repressed by transfer to LL. In contrast, switching from aerobic to microaerobic conditions in LL had no effect on basal CrhR abundance (Figure 3.1C) while constant exposure to HL induced CrhR continuously under microaerobic conditions, an effect that was enhanced by a subsequent shift to aerobic conditions (Figure 3.1D). Finally, microaerobic conditions had no effect on the light response, HL induced while subsequent LL repressed CrhR abundance (Figure 3.1E). The HL-LL/ microaerobic-aerobic results, summarized in Table 3.2, indicate that CrhR expression is primarily redox regulated with a significant input from light.

In order to confirm that high light mediated induction of CrhR was a product of redox regulation, CrhR accumulation was evaluated in the presence of the ETC inhibitors DCMU and DBMIB. The effect of HL on CrhR expression was initially determined at 30oC in the absence of temperature stress (Figure 3.2A). Continuous HL exposure caused a rapid increase in CrhR abundance, a level that remained constantly elevated for the duration of exposure (Figure 3.2A). Culture shift from HL to LL reduced CrhR levels, indicating that HL induction was reversible and thus the factor causing CrhR induction (Figure 3.2A). Association of the redox poise of the ETC with HL induction was provided by the substantial inhibition of HL induction by DCMU oxidation of the ETC downstream of QA (Figure 3.2B). In contrast, enhanced reduction of the ETC by DBMIB upstream of cytochrome $b_6f$ did not affect the HL induction and reversed the anticipated subsequent decrease of CrhR in LL (Figure 3.2C). Analysis of the major photosynthetic parameter, CO2, indicated increased CO2 enhanced CrhR expression an effect that was reversed by return to air levels of CO2 (Figure 3.2D). Oxygen tension did not affect CO2 induction, as similar results were observed in response to altered CO2 abundance under microaerobic conditions (compare Figures 3.2D and 3.2E).

Light quantity enhancement of CrhR abundance prompted further investigation of dark- light transition in conjunction with the presence (20ºC) or absence (30oC) of temperature stress Overexposure of the western blot indicated that shifting *Synechocystis*

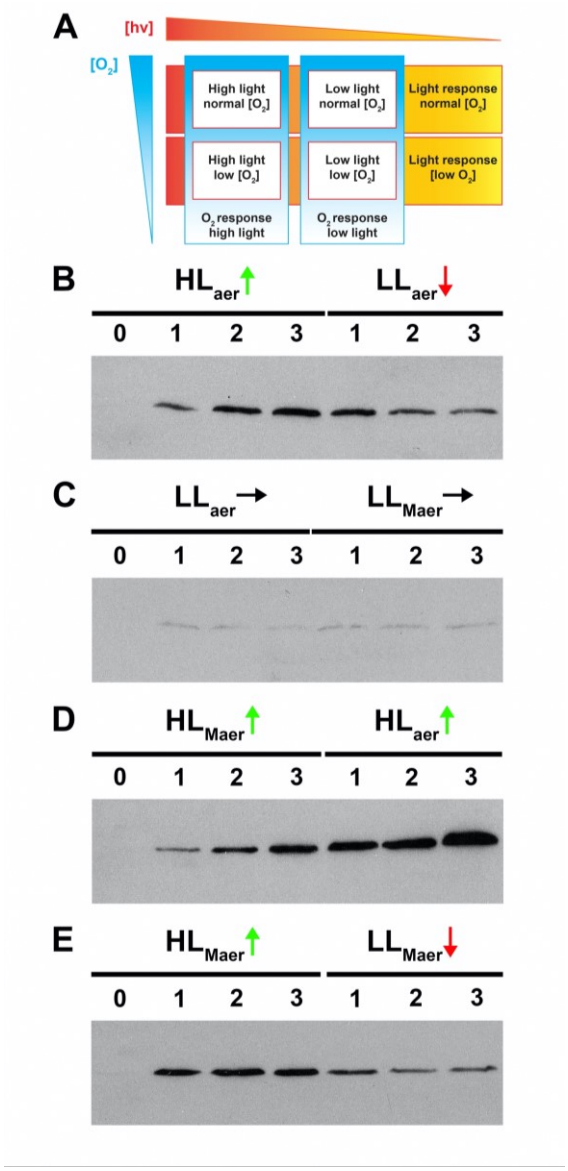



**Figure 3.1. Combinatorial effects of varying oxygen availability and light quantity confirm redox regulation.** Wild type *Synechocystis* cells were grown to mid-log phase at 30°C and exposed to the indicated stresses. HL= High Light (250 µmol $m^{-2}$ $s^{-1}$). LL= Low Light (50 µmol $m^{-2}$ $s^{-1}$). AER= Aerobic conditions. Bubbling with Air. MAER= Microaerobic conditions. Bubbling with 95% $N_2$ + 5% $CO_2$. **(A)** Outline of interlinked experimental setup adapted from Barth et al. (2014). Experimental conditions are outlined in red. Response determined by transitioning between these conditions are displayed in red for light responses, and blue for $O_2$ response. **(B)** Light response with standard $O_2$. Cells grown under LL AER conditions were transferred to HL for 3 h before return to LL for 3 h. **(C)** Light response with low $O_2$. Cells grown under LL AER conditions were sampled for 3 h before moving to MAER for 3 h. **(D)** $O_2$ response with HL. Cells grown under LL AER conditions were transferred to HL + MAER for 3 h before return to AER for 3 h. **(E)** $O_2$ response with LL. Cells grown under LL AER conditions were transferred to HL + MAER for 3 h before return to LL for 3 h.

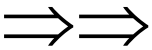
| | Box I (High Light + $O_2$ -> Low Light +$O_2$) | Box II (Low Light + $O_2$ -> Low Light + Anoxia) | Box III (High Light + Anoxia -> High Light + $O_2$) | Box IV (High Light + Anoxia -> Low Light + Anoxia) |
|---|---|---|---|---|
| **Tested Effect** | **Light Effect +/- ROS** | **LL Effect +/- ROS** | **HL Effect +/- ROS** | **Light Effect no ROS** |
| **Observed CrhR Expression Pattern** |  |  |  |  |
| **Light Regulation** |  |  |  |  |
| **ROS Regulation** |  |  |  |  |
| **Redox Regulation** |  |  |  |  |
| **Light Repressed Regulation** |  |  |  |  |
| **"4-Down" Regulation** |  |  |  |  |

**Table 3.2. Summary of oxygen – light manipulation.** A summary of the effects of manipulating oxygen concentration and light intensity on CrhR accumulation from data shown in Figure 1 is provided. The five patterns of protein accumulation observed in *C. reinharditii* are listed on the left (Barth et al., 2014). The experimentally observed pattern of CrhR accumulation is shown with black arrows. CrhR accumulation patterns that match those observed in *C. reinharditii* are depicted as green arrows while results that do not match are shown as red arrows. Vertical, horizontal and down arrows indicate increased, no change and decreased CrhR accumulation, respectively

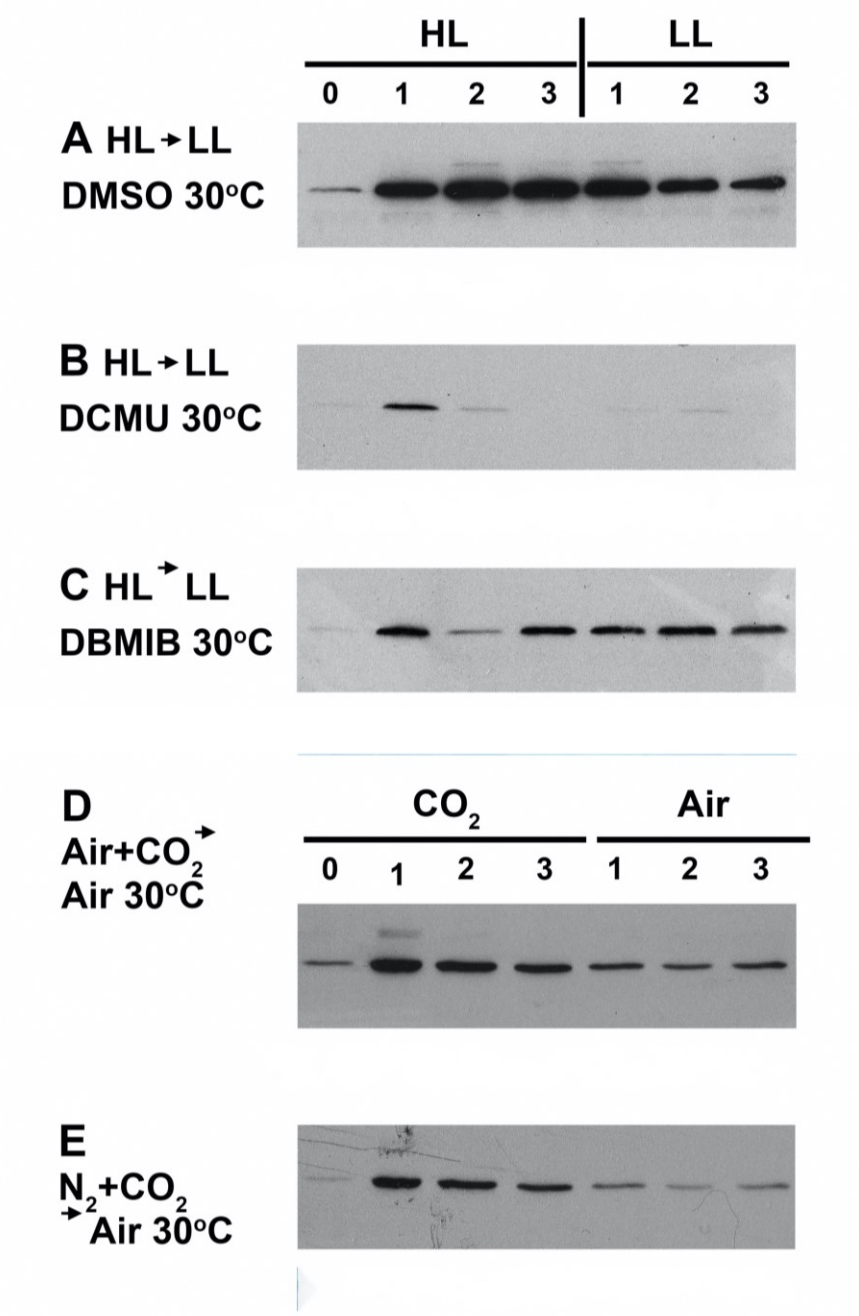



**Figure 3.2 High light and increased $CO_2$ concentration reversibly enhance CrhR accumulation.** Wild type *Synechocystis* cells were grown to mid-log phase at 30°C (T = 0) and the effect of the photosynthetic parameters light quantity and $CO_2$ concentration on CrhR accumulation was analyzed. **(A-C)** Light quantity. Cells grown at LL (50 µmol $m^{-2}$ $s^{-1}$) were transferred to HL (250 µmol $m^{-2}$ $s^{-1}$) for 3 h and then returned to LL for 3 h in the presence of DMSO **(A)**, DCMU **(B)** and DBMIB **(C)**. **(D-E)** Elevated $CO_2$. Wild type *Synechocystis* cells were grown to mid-log phase at 30°C (T = 0) and bubbled with 5% $CO_2$ in air (aerobic) **(D)** or nitrogen (microaerobic) **(E)** for 3 h. Cultures were subsequently bubbled with air (0.04% $CO_2$) for 3 h.

from light to dark to light conditions at 30oC had no substantial effect on the basal level of CrhR that is routinely observed (Figure 3.3A). However, in cultures pre-induced to maximum CrhR abundance by incubation at 20ºC for 3 h (Figure 3.3B, 0 time), the expected repression of CrhR abundance produced by a temperature upshift to 30oC was not observed in the absence of light (Figure 3.3B Dark). Subsequent exposure to light at 30oC initiated the expected decrease in CrhR abundance (Figure 3.3B Light), indicating the requirement for a light signal to initiate CrhR degradation. Repeating the dark-light transitions at 20ºC in the absence (Figure 3.3C) or presence (Figure 3.3D) of CrhR pre-induction produced similar conclusions regarding the importance of a light signal to initiate both CrhR accumulation and degradation. In the absence of pre-induction, the expected low temperature induction of CrhR was not observed. Instead, the basal level of CrhR detected at 30oC decreased extensively in the dark at 20ºC (Figure 3.3C Dark). Subsequent transfer to the light at 20ºC initiated the expected low temperature induction of CrhR (Figure 3.3C Light). Similar results to those shown in Figure 3.3C dark were also observed in cultures pre-induced to maximum CrhR induction at 20ºC, conditions that also caused a steady decline in CrhR abundance in the dark at 30ºC (Figure 3.3D Dark). Again, transfer to the light at 20ºC resulted in CrhR induction (Figure 3.3D Light), similar to the observations shown in Figure 3.3C. Overall, the results suggest that a light signal is required for both low temperature induction and temperature upshift repression of CrhR accumulation.

The results presented above indicate that dark-light transition had a dramatic effect on induction and repression of CrhR accumulation. This analysis was extended to include temperature shift in combination with ETC inhibitors and dark-light transition (Figure 3.4A). To analyze low temperature induction, cells were grown at 30ºC (Figure 3.4 0 time) followed by concurrent transfer to the dark and 20ºC in the presence or absence of ETC inhibitors. As shown in Figure 3.4A DMSO, darkness again inhibited 20ºC induction in wild type cells, an effect that was reversed by transfer to the light. Low temperature induction of CrhR was also not observed in the dark in the presence of either of the ETC inhibitors, DCMU or DBMIB, however DCMU substantially inhibited (Figure 3.4A DCMU Light) while DBMIB enhanced the subsequent low temperature induction in the light (Figure 3.4A DBMIB Light). The incomplete abolition of CrhR expression by DCMU

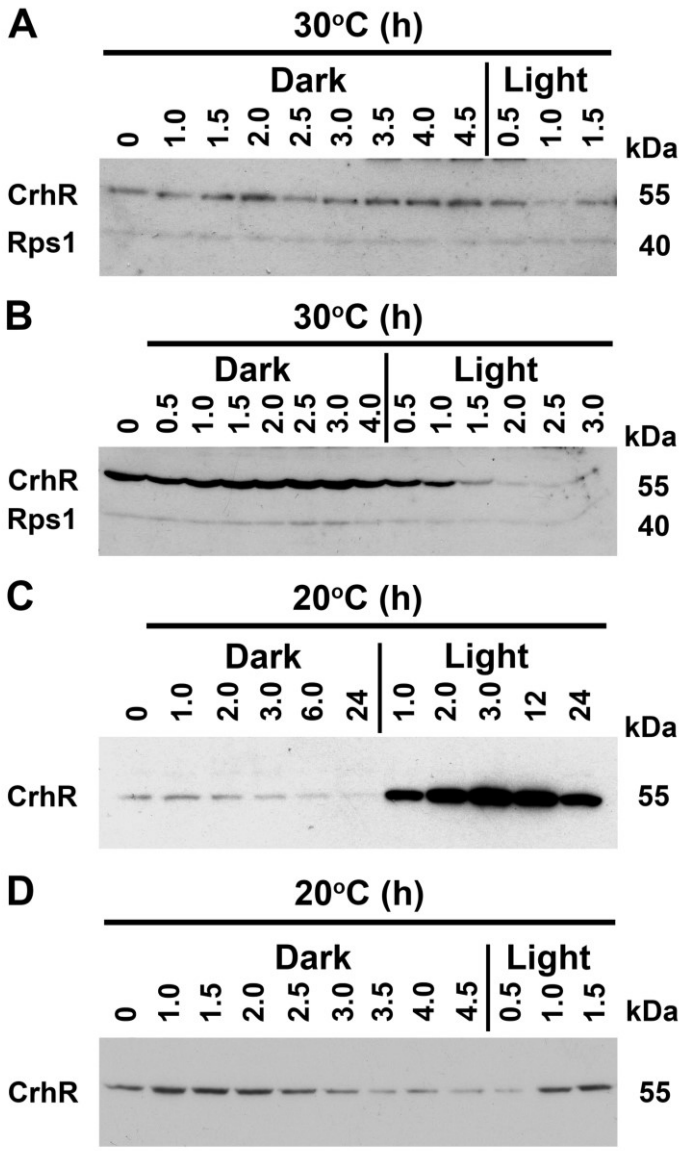



**Figure 3.3 Dark treatment supersedes light signals.** A *Synechocystis* culture was grown to mid-log phase under standard conditions and aliquots subjected to the following treatments. **(A)** Cells were transferred to dark conditions at 30°C for 4.5 h and subsequently exposed to light at 30°C for 1.5 h. **(B)** Cells were pre-induced for maximal CrhR accumulation for 3 h at 20°C (T0) and then transferred to the dark at 30°C for 4.5 h. The culture was then exposed to light for 3 h at 30°C. For **(A)** and **(B)**, western blots were simultaneously probed with antibodies against CrhR (55 kDa) and *E. coli* ribosomal protein S1 (Rps1, 40 kDa) which was used as a control for protein loading. **(C)** Cells were shifted simultaneously to the dark and 20°C for 24 h and then exposed to light at 20°C for 24 h. **(D)** Cells were pre-induced for maximal CrhR accumulation for 3 h at 20°C (T0) and then transferred to the dark at 20°C for 4.5 h. Cells were subsequently transferred to the light at 20°C for 1.5 h.

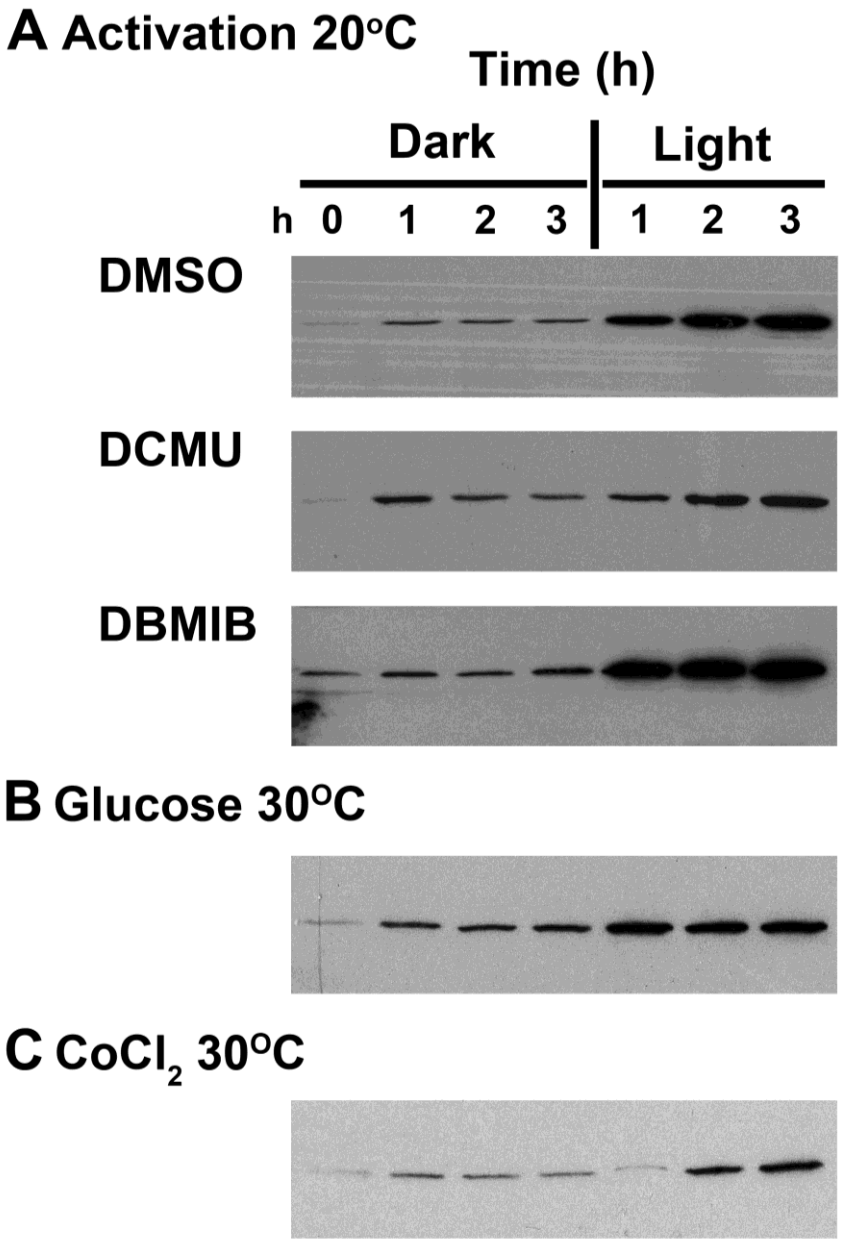



**Figure 3.4. Absence of ETC reduction generates the dark effects.** Wild type *Synechocystis* cells were grown to mid-log phase at 30°C and the effect of dark – light transition associated with stresses that alter the ETC redox potential was analyzed. **(A)** Light – dark transition effect on temperature response. Cells grown at 30oC under standard light conditions were transferred to 20ºC in the dark for 3 h after addition of DMSO, DCMU or DBMIB. Cells were then returned to growth light for 3 h. **(B-C)** Light – dark transition independent of temperature downshift. Cells grown at 30oC under standard light conditions were transferred to the dark for 3 h after addition of glucose **(B)** or $CoCl_2$ **(C)**. Cells were then return to standard growth light for 3 h.

can be attributed to the sub-inhibitory concentration of the inhibitor used in these experiments. Provision with glucose, which should contribute to respiratory electron flow into the PQ pool, marginally enhanced CrhR accumulation in the dark at 30ºC a level that was further enhanced by light exposure (Figure 3.4B Glucose). Cobalt heavy metal stress, previously shown to induce CrhR via its indirect effect on the ETC (Ritter et al., 2019), also required a light signal displaying inhibition of expression in the dark and subsequent induction in the light in the absence of cold stress (Figure 3.4C CoCl2). The cobalt stress results confirm that the dark-light regulation is not specific to temperature stress and further indicate that a light signal is required for induction of CrhR in response to a variety of stresses.

In order to further investigate the nature of the light signal required for low temperature induction of CrhR expression and the previously established redox regulation mechanism, the effect of varying light quality on temperature induction was determined. Cells were incubated in the dark at 30ºC for three h before exposure to either red (662 nm) or blue light (455 nm) at 20ºC. Induction of CrhR occurred normally in the presence of red light (Figure 3.5A "R"); both the rate of induction and the maximum level of CrhR reached occurred to similar levels as observed in white light (Figure 3.5A "RW"). This was not the case in the presence of blue light, where the expected cold induction was not observed (Figure 3.5A "B"). Induction was, however, subsequently restored by exposure of blue-treated cells with white light (Figure 3.5A "BW").

Repression of CrhR by proteolysis, which occurs upon return of cold shocked cells to the normal growth temperature of 30ºC, was also shown to require a light signal for induction (Figure 3.5B). In order to determine whether a specific wavelength of light was required to initiate proteolysis, concurrent treatment with red or blue light and temperature upshift after pre- induction of CrhR at 20ºC for 3 h was performed. In contrast to low temperature induction, temperature upshift induced proteolysis of CrhR was light quality independent, proceeding to basal levels in the presence of both red and blue light (Figure 3.5B). These results suggest that differing mechanisms are responsible for the induction and proteolysis of CrhR, with induction at the protein level being light quality dependent, and proteolysis being contingent on the presence of a separate light signal that is not light quality or redox dependent.

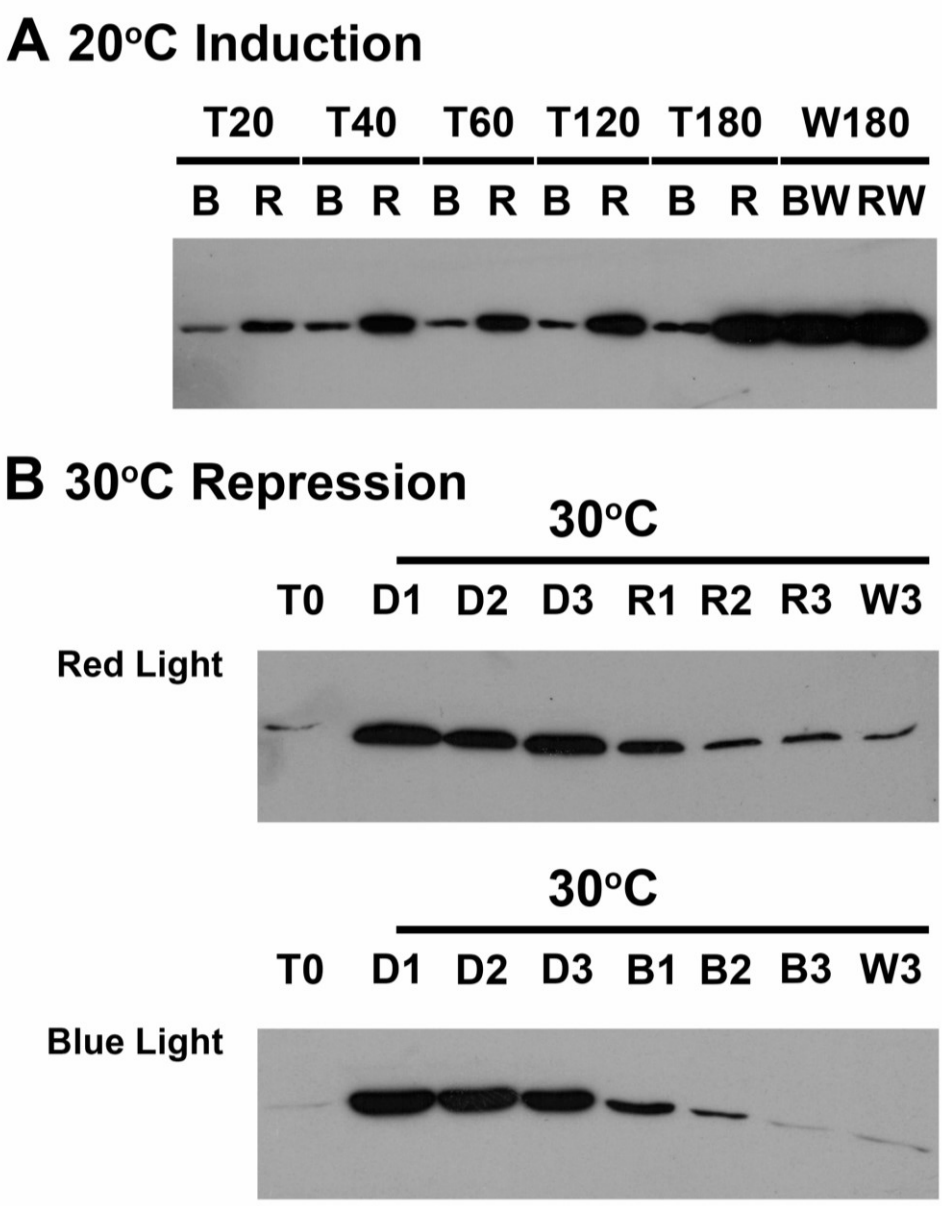



**Figure 3.5. Red light is required for CrhR accumulation.** Wild type *Synechocystis* cells were grown to mid-log phase at 30°C and the effect of varying light quality on CrhR induction and proteolysis was analyzed in conjunction with temperature downshift and upshift. **(A)** Effect of light quality on low temperature induction. Cells grown at 30ºC under standard light conditions were transferred to 20ºC in either blue (B, 455nm) or red (R, 662 nm) light and for 3 h. Cells were then transferred to white light for 3 h (W180). **(B)** Effect of light quality on high temperature repression. Cells were grown at 30ºC under standard light conditions (T0) before pre-induction at 20ºC for 3 h. Cells were then returned to 30ºC in the dark (D) for 3 h before transfer to either red (R) or blue (B) light for 3 h. Finally, cells were incubated in white light for 3 h (W3).

Since there was a distinct difference in the ability of light quality to mediate temperature- responsive induction and repression of CrhR, similar analysis was performed at the transcript level. Enhanced accumulation of *crhR* transcript in response to temperature downshift in the presence of white light occurred much more rapidly than at the protein level (Figure 3.6). In contrast to CrhR protein accumulation, light quality did not significantly affect the low temperature induction of *crhR* transcript accumulation as maximum levels similar to those induced by white light were observed in both red and blue light (Figure 3.6). Blue light did however initially result in a lag in transcript accumulation at 20ºC, as it was significantly lower than values obtained in white and red light after 20 min at 20ºC but was not significantly different at 40 min (Figure 3.6 Blue Light). In the absence of light, low temperature did not enhance *crhR* transcript abundance at 20ºC (Figure 3.6 Dark). An intermediate level of accumulation occurred in the presence of glucose in the dark, with levels significantly higher than observed in dark grown cells, but lower than in any tested light condition (Figure 3.6 Dark+Glc). Cells exposed to all conditions remained metabolically active, as subsequent transfer to white light at 20ºC induced transcript accumulation to similar levels (Figure 3.6 White Light ON).

The ability of blue light to induce cold-shock mediated expression of *crhR* transcript, but not protein, suggests the involvement of a post-translational regulatory mechanism. Using inhibitors that differentially affected translational initiation or elongation, the requirement for *de novo* protein synthesis was assessed in cold-stressed wild type and cells lacking functional CrhR RNA helicase activity, $crhR_{TR}$. The expected basal levels of *crhR* transcript and protein at 30ºC are detected in wild type cells (Figure 3.7A 30ºC). This basal expression differs significantly from the enhanced level of transcript observed upon temperature downshift in wild type cells (Figure 3.7A 20ºC). Transcript and protein accumulation were differentially affected depending on the mode of action of the translational inhibitor (Figure 3.7A Wild type). The presence of inhibitors of translation elongation negated, while inhibition of translation initiation had no effect on low temperature induction at either the transcript or protein level (Figure 3.7A *crhR* and CrhR). Furthermore, it is of note that two *crhR* transcripts were differentially detected (Figure 3.7A *crhR* and CrhR), with the faster migrating transcript present under all conditions and generated the basal level of CrhR protein (Figure 3.7A Wild type). The

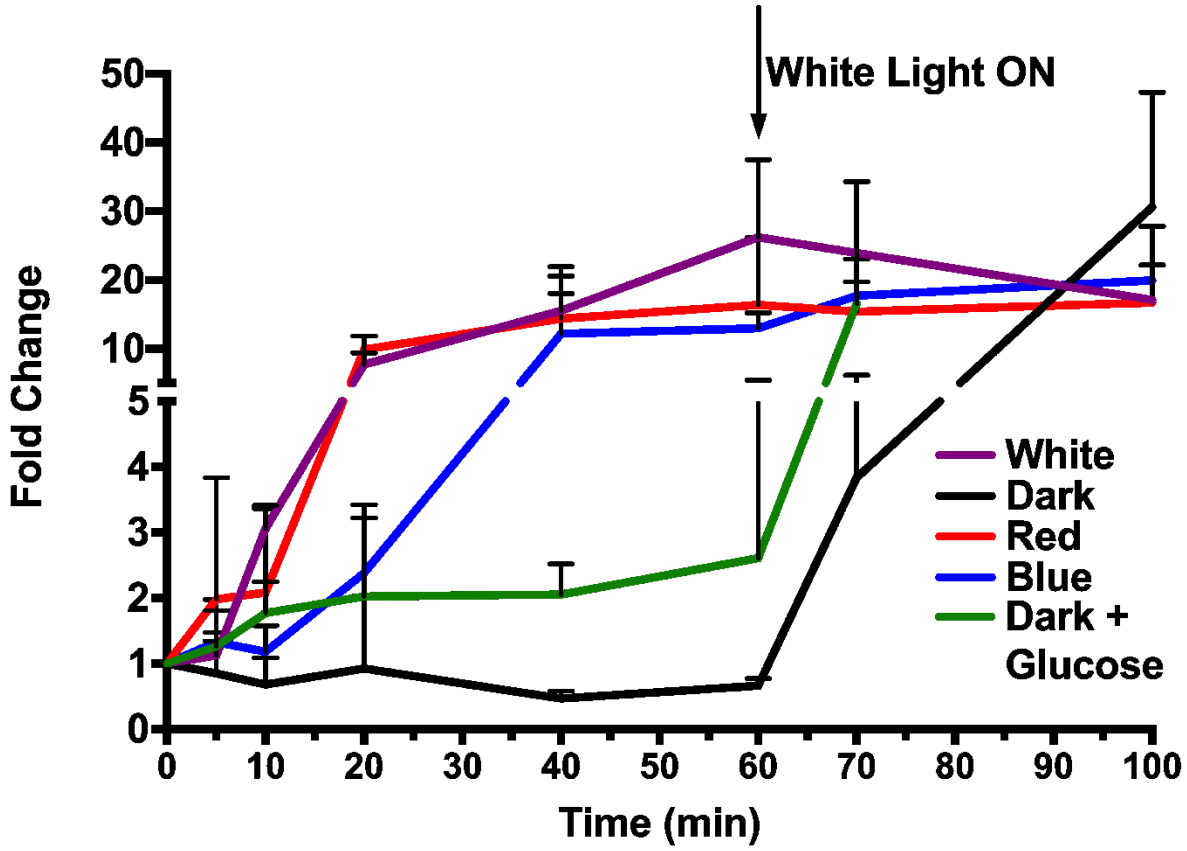



**Figure 3.6. Reduction of the ETC is required for *crhR* transcript accumulation as detected by qPCR.** Wild type *Synechocystis* cells were grown to mid-log phase at 30°C and the effect of varying light quality on *crhR* transcript accumulation was analyzed in conjunction with temperature downshift. Aliquots were transferred to 20ºC in the absence (Black line) or presence of glucose (green line), or with continued illumination with white light (purple line), blue light (blue line), red light (red line) for 60 min. cultures were then transferred to white light for 40 min. Levels were normalized to basal levels at 30oC in white light. The data was obtained from two biological replicates. Error bars represent standard deviation.

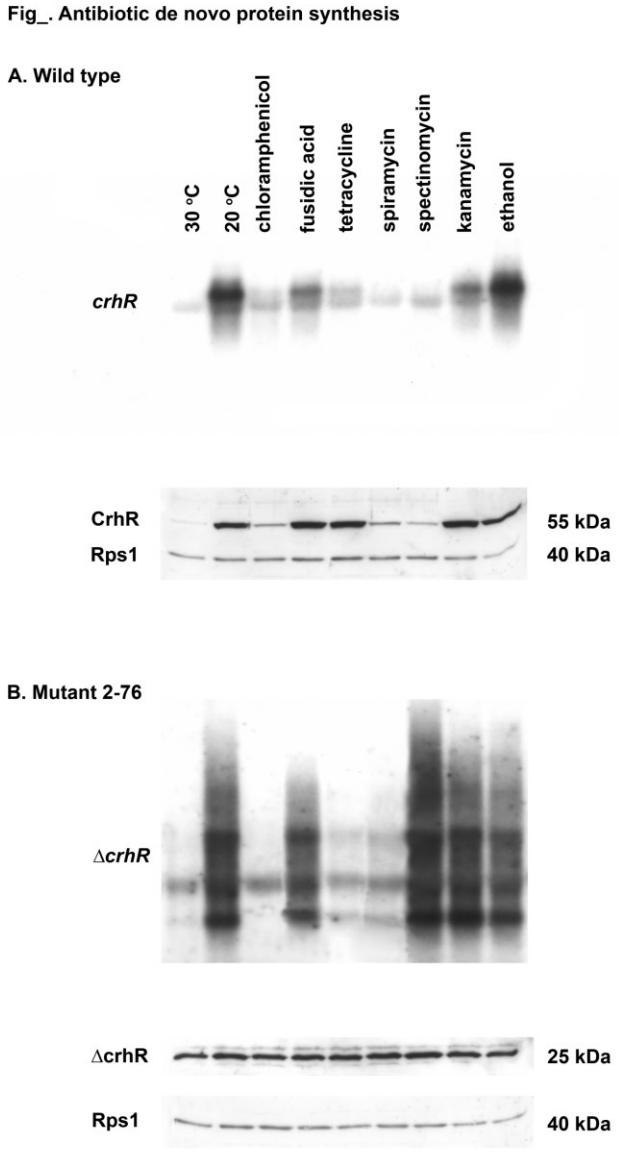



**Figure 3.7. *crhR* translation is pre-initiated at 30°C.** Wild type **(A)** and *crhR*$_{TR}$ **(B)** *Synechocystis* cells were grown to mid-log phase at 30°C. Indicated antibiotics (150 mg/ml) were added and incubation continued for an additional 1 h at 30°C to fully inhibit *de novo* protein synthesis. Cultures were transferred to 20°C and incubated for 1 h for maximum induction of CrhR synthesis. Samples for RNA and soluble protein extraction were processed for northern and western analyses as described previously (Rosana et al., 2012b). *crhR* transcript was detected from total RNA probed with a 93 bp *Hinc*II-*Sac*II internal fragment of *crhR* (Rosana et al., 2012b). Western blots were simultaneously probed with antibodies against CrhR (55 kDa) and *E. coli* ribosomal protein S1 (Rps1, 40 kDa) which was used as a control for protein loading. accumulation only occurred to a maximum level irrespective of transcript abundance (Figure 8A Wild type).

slower migrating transcript only accumulated under conditions that enhanced CrhR protein abundance. In sharp contrast, the lack of functional CrhR RNA helicase activity dramatically altered both transcript and protein accumulation (Figure 3.7B *crhR*$_{TR}$). The basal level of a single *crhR* transcript was enhanced at 30ºC in the *crhR*$_{TR}$ mutant while accumulation of four stable transcripts was induced in the presence of translation initiation inhibitors at 20ºC (Figure 3.7B *crhR*$_{TR}$). Again, as observed in wild type cells in which CrhR was induced (Figure 3.7B CrhR), CrhR protein accumulated to identical levels in *crhR*$_{TR}$ cells irrespective of transcript abundance, inhibitor presence or temperature (Figure 3.7B *crhR*$_{TR}$).

## 3.5 Discussion

In order to survive, obligate photoautotrophic organisms including many cyanobacteria, depend on the ability to sense and respond to light, whose harvesting provides all of the energy required to form carbon skeletons by photosynthetic carbon fixation. To accommodate the diverse range of light regimes cyanobacteria experience in nature, they have evolved a range of light-sensing systems that modulate gene expression using diverse mechanisms. Here we show that three distinct light signals affect CrhR expression at discrete points, as modeled in Figure 3.8. One signal is associated with light induced reduction of PQ that enhances *crhR* transcript accumulation (Figure 3.8A). The second is a wavelength dependent light signal required for translation and appears to be facilitated by a member of the *Synechocystis* phytochrome superfamily (Figure 3.8B). The third signal is also contingent on light per se but is wavelength- and redox-responsive independent, being required for the initiation of CrhR proteolysis in the absence of stress (Figure 3.8C). Although the results presented here are in contrast to data suggesting that cold induction of *crhR* transcript accumulation occurred in the dark (Mironov et al., 2012; Mironov et al., 2014), they agree with data indicating that light was required for CrhR protein accumulation (Kujat and Owttrim, 2000; Chen et al., 2018). Overall, CrhR appears to play a role in maintaining homeostasis during photosynthesis in response to conditions that could result in over-reduction of the ETC (Rosana et al., 2012a; Ritter et al., 2019).

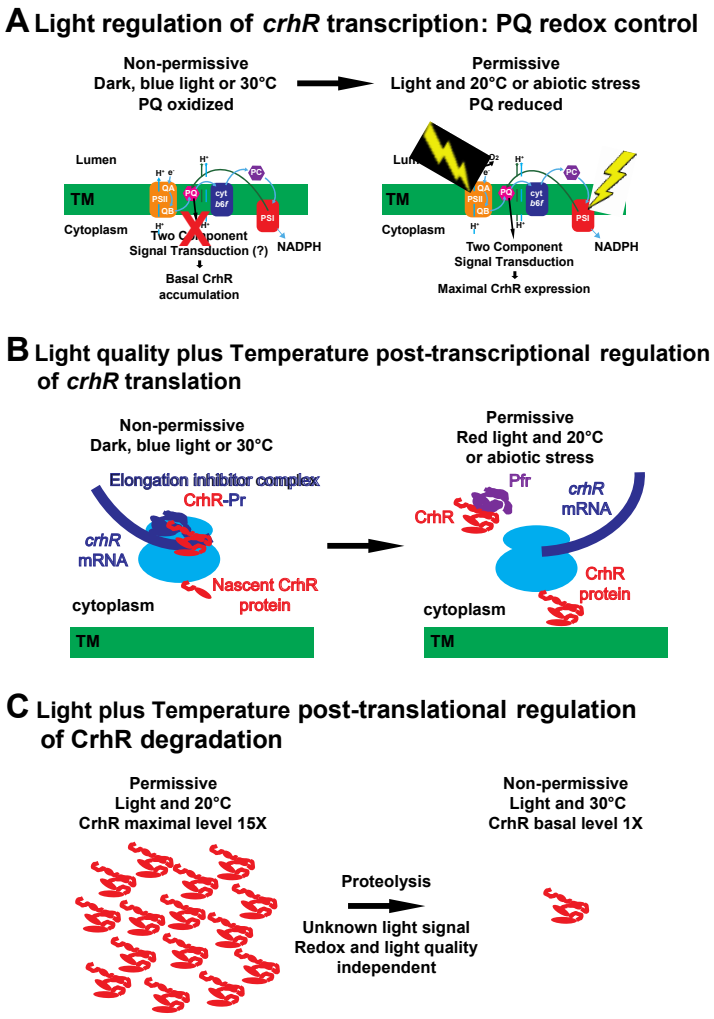



**Figure 3.8. Model: Discrete light signals regulate *crhR* expression. (A-C)** Three discrete light signals regulate *crhR* expression at distinct points. **(A)** Light driven redox regulation. Under non-permissive conditions when CrhR is not required (dark, blue light, or 30°C) the relatively oxidized PQ pool does not activate a two-component signal transduction chain leading to enhanced *crhR* expression. This unknown signal transduction pathway is activated by permissive conditions (light and 20°C or abiotic stress) yielding *crhR* expression. **(B)** Light quality regulation of CrhR accumulation. Conditions under which CrhR is not required (dark, blue light or 30°C) we propose that translation has initiated on *crhR* transcripts however elongation has halted, potentially involving CrhR and/or Pr binding. Transfer to permissive conditions where CrhR is required (red light and 20°C) relives the translational block allowing translation elongation and full length CrhR production. In response to dark-light transfer, conversion of Pr-CrhR to Pfr-CrhR or binding of activated Pfr to CrhR could remove the translational block. **(C)** Light-dependent proteolysis. A redox- and light quality-independent light signal is required for induction of CrhR proteolysis in response to temperature upshift from 20°C to the non-permissive condition, 30°C.

*3.5.1 The role of light mediated alteration of the PQ redox poise*

The importance of light sensing and signaling for *crhR* expression was exemplified by the diversity of light parameters that influenced multiple stages of *crhR* expression. Utilization of an interlinked experimental system allowed us to assess the individual contributions of light, redox and ROS on gene expression (Barth et al., 2014) and confirmed previous evidence (Kujat and Owttrim, 2000; Ritter et al., 2019) that the redox poise of the ETC was the primary driver of *crhR* expression with light not unexpectedly playing an important role (Figure 3.8A). This analysis further demonstrated the interconnection between light and redox regulatory pathways as CrhR exhibited a protein expression pattern termed redox, associated with ROS detoxification, redox regulation/balance and redox signaling (Barth et al., 2014). Light quantity regulation of CrhR expression was also associated with the redox potential of PQ, as DCMU resulted in a reduced level of CrhR accumulation in response to high light. In addition, the essential contribution of the PQ redox status for *crhR* expression was emphasized by glucose partially rescuing transcript and protein accumulation in the dark. Thus, a signal derived from the redox status of PQ and not light per se is required for *crhR* expression.

*3.5.2 light quality regulates crhR translation*

Light quality was also shown to be crucial for *crhR* expression as transcript accumulated in both red and blue light in response to cold stress, while protein only accumulated in red light. This suggests that red-light activation of a member of the phytochrome superfamily protein, encoded in *Synechocystis* (Wiltbank and Kehoe, 2019), was required to permit *crhR* translation but not transcription in response to abiotic stress. This requirement of a permissive light signal for translation is not unique to CrhR, as over half of the tested cold responsive proteins in *Synechocystis* responded similarly to CrhR (Chen et al., 2018). However, examples of post-transcriptional control of expression in response to light quality are rare (Paik et al., 2012).

Phytochrome is a major regulator of gene expression in both higher plants and cyanobacteria where it controls key physiological processes including chromatic acclimation, phototaxis, gene expression and cellular growth rate (Choi et al., 1999; Wilde et al., 2002; Hubschmann et al., 2005; Wiltbank and Kehoe, 2019). We propose that

regulation of *crhR* translation occurs via a phytochrome-mediated mechanism related to that controlling protochlorophyllide reductase (PORA) expression in *Arabidopsis thaliana* (Figure 3.8B, Paik et al., 2012). PORA translation is negatively regulated in the light via an unknown mechanism involving interaction between Pnt1, an RNA binding protein constitutively bound to the *pora* 5' UTR, and light-activated phytochrome (Pfr) (Paik et al., 2012). Dark relieves this inhibition, causing conversion of phytochrome to its inactive form (Pr) and dissociation from Pnt1 (Paik et al., 2012). We envisage that CrhR binds its own transcript under non-permissive conditions, dark, blue light or high temperature, to inhibit translation elongation, acting similarly to Pnt1 (Figure 3.8B). Relief of inhibition would require interaction of the translation elongation inhibitory protein, potentially CrhR, with an activated photoreceptor belonging to the phytochrome superfamily to allow translation under permissive conditions, either cold stress or red light (Figure 3.8B). Evolution of the light quality requirement for *crhR* expression is likely associated with the fact that although phycobilisomes perform the majority of light harvesting in cyanobacteria they absorb minimally at wavelengths below 495 nm, thus reduction of PQ will be minimal in blue light (Grossman et al., 1993; Luimstra et al., 2018). The lack of CrhR translation in blue light is therefore expected, as *crhR* transcript accumulation is dependent on enhanced reduction of PQ which is driven by electrons derived from light-activated water splitting (Kujat and Owttrim, 2000; Ritter et al., 2019).

The phytochrome model shown in Figure 3.8B, was also supported by the observation that low temperature induction of *crhR* continued in the presence of antibiotics that inhibit translation initiation while elongation inhibitors blocked *crhR* expression. This suggests that *crhR* translation was pre-initiated at the non-permissive condition, 30ºC, but elongation was stalled potentially by CrhR binding since translational control was not observed in the absence of functional CrhR RNA helicase activity. Transfer to the permissive condition, 20ºC, could remove CrhR via an unknown mechanism thus alleviating the inhibition and allowing elongation to proceed. These results again support the conclusion that a phytochrome-mediated mechanism regulates the post-translational accumulation of CrhR protein (Figure 3.8B). CrhR association with the thylakoid membrane (Rosana et al., 2016) relates the mechanism proposed in Figure 3.8B with regulation of D1 expression in *Synechocystis* where a stalled D1-polysome complex forms

in the cytoplasm in the dark. An unspecified light signal targets the initiated complex to the thylakoid membrane where translation is completed (Tyystjärvi et al., 2001). Together, the data indicate that a variety of mechanisms can impart translational control in response to light in cyanobacteria.

### *3.5.3 A light signal regulates CrhR proteolysis*

Repression of a light dependent response is also required to maintain cellular/plant fitness by regulation of stomatal development, phytochrome responses (Lee et al., 2017; Park et al., 2018) and D1 protein turnover and phycobilisome degradation and in cyanobacterial systems (Boehm et al., 2012; Baier et al., 2014). CrhR abundance is also regulated at the post-translational level, as abiotic stress enhanced CrhR expression is alleviated by conditional proteolysis in response to upshift to non-permissive temperatures (Tarassova et al., 2014, Ritter et al., 2019). The data presented here indicated that induction of CrhR proteolysis also required a general light signal, as it did not occur in dark treated cells. Proteolysis also proceeded independently of redox regulation, since degradation occurred normally in the presence of various ETC inhibitors (Figure 3.8C). In addition, the proteolytic repression of CrhR protein accumulation in response to temperature upshift was not light quality dependent, since CrhR degradation was observed under all wavelengths tested (Figure 3.8C).

### *3.5.4 Dark incubation supersedes all three light signals*

Finally, the overall importance of light for *crhR* expression was demonstrated by the observation that dark conditions superseded all three light signals, emphasizing the requirement for CrhR catalyzed RNA helicase activity in growth conditions that promote photosynthesis. Gene expression in response to dark-light transition is regulated by the circadian clock in cyanobacteria (Köbler et al., 2018; Welkie et al., 2018). It will be of interest to determine the extent to which the circadian clock is associated with *crhR* expression in *Synechocystis*.

In summary, we show that a complex series of discrete light sensing and signaling mechanisms contribute to the regulation of *crhR* expression at distinct levels of expression. These findings are unique, as while gene regulation by various light parameters is well

characterized, examples of light regulation of individual genes at multiple levels has not been reported. These mechanisms, especially the pre-initiation of *crhR* translation under non-permissive conditions, would allow for the rapid adjustment of CrhR abundance aiding acclimation to the range of light and abiotic stress conditions *Synechocystis* continuously encounters in the natural habitat. Elucidation of the mechanisms by which these light and redox signals are sensed and the signal transduction pathways that are activated to alter CrhR expression remain outstanding questions.

## 3.6 References


Alfonso A, Perewoska I, Kirilovsky D. 2000. Redox Control of *psbA* Gene Expression in the Cyanobacterium *Synechocystis* PCC 6803. Involvement of the Cytochrome $b_6f$ Complex. Plant Physiology 122, 505-515.

Anderson SL, McIntosh L. 1991. Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: A blue-light-requiring process. Journal of Bacteriology 173, 2761–2767.

Baier A, Winkler W, Korte T, Lockau W, Karradt A. 2014. Degradation of phycobilisomes in *Synechocystis* sp. PCC6803: evidence for essential formation of an NblA1/NblA2 heterodimer and its codegradation by A Clp protease complex. Journal of Biological Chemistry 289, 11755–11766.

Balasubramanian R, Shen G, Bryant DA, Golbeck JH. 2006. Regulatory roles for IscA and SufA in iron homeostasis and redox stress responses in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Journal of Bacteriology 188, 3182–3191.

Barth J, Bergner SV, Jaeger D, Niehues A, Schulze S, Scholz M, Fufezan C. 2014. The interplay of light and oxygen in the reactive oxygen stress response of *Chlamydomonas reinhardtii* dissected by quantitative mass spectrometry. Molecular and Cellular Proteomics 13, 969–989.

Bezy RP, Wiltbank L, and Kehoe DM. 2011. Light-dependent attenuation of phycoerythrin gene expression reveals convergent evolution of green light sensing in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 108, 18542–18547.

Boehm M, Yu J, Krynicka V, Barker M, Tichy M, Komenda J, Nixon PJ, and Nield J. 2012. Subunit organization of a *Synechocystis* hetero-oligomeric thylakoid FtsH complex involved in photosystem II repair. Plant Cell 24, 3669–3683.

Bogorad L, and Park YM. 1999. Photomovement of the gliding cyanobacterium *Synechocystis* sp. PCC 6803. Photochemisty and Photobiolology 70, 95–102.

Calzadilla PI, Zhan J, Sétif P, Lemaire C, Solymosi D, Battchikova N, Wang Q, Kirilovsky D. 2019. The cytochrome $b_6f$ complex is not involved in cyanobacterial state transitions. Plant Cell 31, 911–931.

Campbell EL. 2015. Genetic analysis reveals the identity of the photoreceptor for phototaxis in hormogonium filaments of *Nostoc punctiforme*. Journal of Bacteriology 197, 782–791.

Chen W, Fang L, Huang X, Ge H, Wang J, Wang X, Zhang Y, Sui N, Xu W, and Wang Y. 2018. Systematic identification of light-regulated cold-responsive proteome in a model cyanobacterium. Journal of Proteomics 179, 100–109.

Conradi FD, Zhou RQ, Oeser S, Schuergers N, Wilde A, and Mullineaux CW. 2019. Factors controlling floc formation and structure in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Journal of Bacteriology 201, 00344-19.

Fujisawa T, Masuda, S. 2018. Light-induced chromophore and protein responses and mechanical signal transduction of BLUF proteins. Biophysics Reviews 10, 327–337.

Georg J, Rosana ARR, Chamot D, Migur A, Hess WR, Owttrim GW. 2019. Inactivation of the RNA helicase CrhR impacts a specific subset of the transcriptome in the cyanobacterium *Synechocystis* sp. PCC 6803. RNA Biology 16, 1205–1214.

Grossman AR, Schaefer MR, Chiang GG, Collier JL. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiology Reviews. 57, 725– 749.

Gutu A, Nesbit AD, Alverson AJ, Palmer JD, Kehoe DM. 2013. Unique role for translation initiation factor 3 in the light color regulation of photosynthetic gene expression. Proceedings of the National Academy of Sciences of the United States of America USA 110, 16253–16258.

Hirano M, Satoh K, Katoh, S. 1980. Plastoquinone as a common link between photosynthesis and respiration in blue-green alga. Photosynthesis Research 1, 149–162.

Hubschmann T, Yamamoto H, Gieler T, Murata N, Borner T. 2005. Red and far- red light alter the transcript profile in the cyanobacterium *Synechocystis* sp PCC 6803: impact of cyanobacterial phytochromes. FEBS Letters 579, 1613–1618.

Ibrahim IM, Puthiyaveetil S, Allen JF. 2016. A two-component regulatory system in transcriptional control of photosystem stoichiometry: Redox-dependent and sodium ion-dependent phosphoryl transfer from cyanobacterial histidine kinase Hik2 to response regulators Rre1 and RppA. Frontiers in Plant Science 12, 196–212.

Joshua S, Mullineaux CW. 2004. Phycobilisome diffusion is required for light-state transitions in cyanobacteria. Plant Physiolology 135, 2112–2119.

Köbler C, Schultz SJ, Kopp D, Voigt K, Wilde A. 2018. The role of the *Synechocystis* sp. PCC 6803 homolog of the circadian clock output regulator RpaA in day- night transitions. Molecular Microbiology 110, 847–861.

Kujat SL, Owttrim GW. 2000. Redox-Regulated RNA Helicase Expression. Plant Physiology 124, 703-714.

Lee JH, Jung JH, Park CM. 2017. Light inhibits COP1-mediated degradation of ICE transcription factors to induce stomatal development in Arabidopsis. Plant Cell. 29, 2817–2830.

Linder P, Owttrim GW. 2009. Plant RNA helicases: linking aberrant and silencing RNA. Trends in Plant Science 14, 344–352.

Linder P, Jankowsky E. 2011. From unwinding to clamping — the DEAD box RNA helicase family. Nature Reviews Molecular Cell Biology, 12, 505–516.

Locato V, Cimini S, De Gara, L. 2018. ROS and redox balance as multifaceted players of cross-tolerance: epigenetic and retrograde control of gene expression. Journal of Experimental Botany. 69, 3373–3391.

Luimstra VM, Schuurmans JM, Verschoor AM, Hellingwerf KJ, Huisman J, Matthijs HCP. 2018. Blue light reduces photosynthetic efficiency of cyanobacteria through an imbalance between photosystems I and II. Photosynthesis Reseach. 138, 77–189.

Mironov KS, Sidorov RA, Trofimova MS, Bedbenov VS, Tsydendambaev VD, Allakhverdiev SI., Los DA. 2012. Light-dependent cold-induced fatty acid unsaturation, changes in membrane fluidity, and alterations in gene expression in *Synechocystis*. Biochimica et Biophysica Acta 1817, 1352–1359.

Mironov KS, Sidorov RA, Kreslavski VD, Bedbenov VS, Tsydendambaev VD, and Los DA. 2014. Cold-induced gene expression and ω(3) fatty acid unsaturation is controlled by red light in *Synechocystis*. Journal of Photochemistry and Photobiology 137, 84–88.

Montgomery BL. 2007. Sensing the light: photoreceptive systems and signal transduction in cyanobacteria. Molecular Microbiology 64, 16–27.

Mullineaux CW, Allen JF. 1986. The state 2 transition in the cyanobacterium *Synechococcus* 6301 can be driven by respiratory electron flow into the plastoquinone pool. FEBS Letters. 205, 155-160.

Mullineaux CW, Allen JF. 1990. State 1-State 2 transitions in the cyanobacterium *Synechococcus* 6301 are controlled by the redox state of electron carriers between Photosystems I and II. Photosynthesis Research 23, 297–311.

Mullineaux CW, Emlyn-Jones D. 2005. State transitions: an example of acclimation to low-light stress. Journal of Experimental Botany 56, 389–393.

Owttrim, G.W. 2012. RNA helicases in cyanobacteria: biochemical and molecular approaches. Methods in Enzymology 511, 385–403.

Owttrim, G.W. 2013. RNA helicases: diverse roles in prokaryotic response to abiotic stress. RNA Biology 10, 96–110.

Park E, Kim Y, Choi, G. 2018. Phytochrome B Requires PIF Degradation and Sequestration to Induce Light Responses across a Wide Range of Light Conditions. Plant Cell 30, 1277–1292.

Paik I, Yang S, Choi, G. 2012. Phytochrome regulates translation of mRNA in the cytosol. Proceedings of the National Academy of Sciences of the United States of America USA 109, 335–1340.

Pinto F, Pacheco CC, Ferreira D, Moradas-Ferreira P, Tamagnini P. 2012. Selection of suitable reference genes for RT-qPCR analyses in cyanobacteria. PLoS ONE 4, e34983.

Ritter SPA, Lewis AC, Vincent S., Lo LL, Cunha APA, Chamot D, Ensminger I, Espie GS. Owttrim GW. 2019. Evidence for convergent sensing of multiple abiotic stresses in cyanobacteria. Biochemica et Biophysica Acta. 1864, 129462.

Rockwell NC, Lagarias JC. 2017. Phytochrome diversification in cyanobacteria and eukaryotic algae. Current Opinions in Plant Biology 37, 87–93.

Rosana AR, Chamot D, Owttrim GW. 2012a. Autoregulation of RNA helicase expression in response to temperature stress in *Synechocystis* sp. PCC 6803. PLoS ONE 7, e48683.

Rosana AR, Ventakesh M, Chamot D, Patterson-Fortin LM, Tarassova O, Espie GS, Owttrim GW. 2012b. Inactivation of a low temperature-induced RNA helicase in *Synechocystis* sp. PCC 6803: physiological and morphological consequences. Plant and Cell Physiology 53, 646–658.

Rosana A.R, Whitford DS, Fahlman RP, and Owttrim GW. 2016. Cyanobacterial RNA helicase CrhR localizes to the thylakoid membrane region and cosediments with degradosome and polysome complexes in *Synechocystis* sp. strain PCC 6803. Journal of Bacteriology 198, 2089–2099.

Tarassova OS, Chamot D. Owttrim GW. 2014. Conditional, temperature-induced proteolytic regulation of cyanobacterial RNA helicase expression. Journal of Bacteriology 193, 1560– 1568.

Tyystjärvi T, Herranen M, Aro EM. 2001. Regulation of translation elongation in cyanobacteria: membrane targeting of the ribosome nascent-chain complexes controls the synthesis of D1 protein. Molecular Microbiology 240, 476–484.

Welkie DG, Rubin BE, Chang YG, Diamond S, Rifkin SA, LiWang A, Golden SS. 2018. Genome-wide fitness assessment during diurnal growth reveals an expanded role of the cyanobacterial circadian clock protein KaiA. Proceedings of the National Academy of Sciences of the United States of America USA 115, 7174–E7183.

Westermark S, Steuer R. 2016. Toward multiscale models of cyanobacterial growth: A modular approach. Frontier in Bioengineering and Biotechnology 4, 95.

Wilde A, Fiedler B, Borner T. 2002. The cyanobacterial phytochrome Cph2 inhibits phototaxis towards blue light. Molecular Microbiology 44, 981–988.

Wiltbank LB, Kehoe DM. 2016. Two cyanobacterial photoreceptors regulate photosynthetic light harvesting by sensing teal, green, yellow and red light. mBio 7, e02130– 15.

Wiltbank LB, Kehoe DM. 2019. Diverse light responses of cyanobacteria mediated by phytochrome superfamily photoreceptors. Nature Reviews Microbiology 17, 37–50.

# Chapter 4: Redox poise of the ETC is sensed through $Q_B$ and transmitted by Hik8-RpaA-RpaB signaling in *Synechocystis* PCC 6803

## 4.1 Abstract

In order to sense and respond to varying environmental conditions, the model cyanobacterium *Synechocystis* PCC 6803 utilizes an expanded complement of two-component signaling mechanisms. Although classically characterized as responsive to a single activating stress, it is clear that a number of two-component signaling systems respond to multiple abiotic stresses. A prime example of this is the association of multiple systems which alter gene expression in response to alterations in the redox status of the ETC surrounding PQ. However, the specific site within the ETC which is sensed and the downstream signaling proteins which enact corresponding changes in redox mediated gene regulation remain unidentified. Through provision with exogenous quinones which accept electrons from different sites of the ETC, we demonstrate that the redox poise of $Q_B$ and not PQ is the most likely position of sensing required to regulate expression of the RNA helicase, CrhR. Through systematic analysis of both loss of function mutants and overexpression constructs of various two-component signaling proteins, we demonstrate that expression of CrhR is partially inhibited by deletion of the response regulator *rpaA*. The partial effect suggests functional compensation through interactions at the protein and promoter level with RpaB or a similarly redundant RR also regulated by Hik8. Investigation of the CrhR regulatory mechanism which responds to changes in the redox poise of $Q_B$ and are detected by Hik8 expands the compliment of redox responsive signal transduction pathways in *Synechocystis* and links *crhR* expression with light - dark and circadian clock regulation.

## 4.2 Introduction

In the free-living model cyanobacterium, *Synechocystis* sp. PCC 6803, the majority of responses to specific environmental signals are perceived and initiated by two-component signaling systems. These consist of a sensory histidine kinase (HK) that interacts with from 1-3 cognate response regulators (RRs) which elicit changes in downstream gene expression. *Synechocystis* encodes 47 HKs and 42 RRs, presumably a reflection of the diversity of environmental conditions experienced in the natural habitat (Mizuno et al., 1996; Marin et al., 2003). HKs are characterized by the presence of both HisKA (dimerization and phosphoacceptor) and HATPase (histidine kinase ATPase) domains, while the RRs contain an aspartate containing receiver domain (Ashby and Houmard, 2006). Upon activation by an environmental stimulus, the HisKA and HATPase domains function to facilitate auto-phosphorylation. The activated HK phosphorylates an aspartate residue in the receiver domain of a RR, resulting in alteration of target gene expression. These systems are widely conserved in prokaryotes including three HKs, Hik2, Hik33, and Hik34, that are conserved across cyanobacteria (Ashby and Houmard, 2006). Homologs also occur in plants, fungi and protists, for example a homolog of *hik2*, Chloroplast Sensor Kinase (CSK), is conserved across chloroplasts and algae suggesting a crucial functional role (Puthiyaveetil et al., 2008).

Although *hik2* is an essential gene in *Synechocystis*, *csk* knockouts in *Arabidopsis* fail to properly regulate gene expression associated with photosynthetic reaction centers (Puthiyaveetil et al., 2008). This system is analogous to state transition in cyanobacteria, the process by which energy is distributed between PSI and PSII via reallocation of phycobilisomes (Mao et al., 2002). The redox poise of plastoquinone (PQ) has been proposed to control state transition in *Synechocystis* via an unidentified signaling system (Joshua and Mullineux, 2004). Hik2, originally identified as an osmoregulator, has since been proposed to respond to the redox poise of the PQ pool to regulate gene expression (Paithoonrangsarid et al., 2004). A similar regulatory mechanism occurs in chloroplasts, with binding and release of $PQH_2$ at the $Q_o$ site of cyt $b_6f$ being sensed by CSK, leading to state transition (Zito et al., 1999). The mechanism of signaling responsible for state transition in cyanobacteria remains unclear but varies from higher plants as cytochrome $b_6f$

is not involved and Ser/Thr kinases are not required for signal transduction (Calzadilla et al., 2019).

Signal transduction downstream of Hik2 has been demonstrated to proceed through both Rre1 and Rre33 (Ibrahim et al., 2016). In addition, Rre33 was originally characterized as acting downstream of Hik30 to control the redox and high light responsive expression of the PSII gene *psbA* (Li and Sherman, 2000). Rre33 is therefore part of a convergent signaling mechanism, translating inputs from a minimum of two HKs. These results suggest that the redox poise around PQ is sensed by multiple interacting two-component signaling pathway in *Synechocystis* that are affected by a range of environmental stimuli and elicit expression of divergent regulons. Similar to Hik2*,* convergent signaling via Hik33 is responsive to the ETC redox poise in the vicinity of PQ (Ibrahim et al., 2016; Maksimov et al., 2017). Redox regulation by Hik33 depends on membrane fluidity, as the rate of PQ reduction has been shown to be directly affected by fatty acid saturation within the membrane, which in turn effects downstream gene expression (Maksimov et al., 2017).

An alternate point of convergent signaling is facilitated by interaction of Hik33 and Hik8 with the RRs, RpaA and RpaB. Hik33 interacts with both RpaA and RpaB (Mikami et al., 2002). These RRs affect phosphorylation of one another at the protein level, as overexpression of RpaB decreased the ratio of phosphorylated RpaA (Espinosa et al., 2015). Functionally, both RpaA and RpaB bind to conserved high light regulated 1 (HLR1) sites within promoters of light responsive genes, such as the *kaiABC* circadian oscillator. It has been suggested that phosphorylated RpaB acts as a negative regulator during subjective night periods, typically binding at HLR1 elements and preventing transcription (Hanaoka and Tanaka, 2008). However, RpaB has been demonstrated to both enhance and repress expression of the PSI subunit *psaA,* which contains multiple HLR1 sites within the upstream promoter (Takahashi et al., 2010). Whether binding occurs at the downstream or upstream of the HLR1 site determines the corresponding effect on gene expression. The phosphorylation state of RpaA appears to be affected by two separate HKs, with both Hik8 and Hik33-mediated phosphorylation of RpaA activating expression of genes involved in circadian gene expression, such as the aforementioned *kaiABC,* and photosynthesis/metabolism such as the key PSI subunit *psaB* and multiple NADH dehydrogenase subunits (*ndhD2/3,ndhF3)* (Iijima et al., 2015; Köbler et al., 2018) A link

between the RpaA/RpaB system and redox regulation was provided by the observation that overexpression of Hik8 causes misregulation of genes associated with PSI and PSII assembly (Kuwahara et al., 2015). It is clear that the Hik33/RpaB and Hik8/RpaA two component signaling systems facilitate a direct link between the circadian clock and the redox poise of the electron transport chain (ETC). Thus, it is clear that a number of cross-reacting HK-RR systems are associated with sensing of the redox poise of PQ which elicits expression of divergent downstream regulons.

Although the redox poise of PQ has been ascribed to regulate expression of multiple genes (Zito et al., 1999; Kujat and Owttrim 2000; Alfonso et al., 2000; Ibrahim et al., 2016), limited evidence has recently been presented suggesting the redox status of $Q_B$ is actually being sensed (Komenda and Barber, 1995). The first stable electron acceptor within PSII is immobile semiquinone $Q_A$. $Q_A$ has been shown to be capable of reducing diverse quinones other than PQ via the secondary quinone $Q_B$ (Satoh et al., 1995). The redox poise of PQ has recently been proposed to be under strict control even when treated with DCMU and DBMIB, raising questions of how small changes in the proportions of oxidized and reduced forms of a freely diffusible PQ pool could be evaluated by a HK to initiate downstream gene regulation (Schuurmans *et al.,* 2017). Utilizing *Synechocystis* mutants Δ*psbO* and Δ*psbH*, which act on the donor and acceptor sides of PSII respectively, Komenda and Barber (1995) showed that D1 protein turnover is impeded in the presence of increased $Q_B$ photodamage. Since D1 turnover is responsive to the redox poise of the ETC, this potentially suggests that the redox poise of $Q_B$, and not PQ, may be the critical element which facilitates redox signaling.

In order to deal with excess excitation pressure around PQ, *Synechocystis* also utilizes multiple flavodiiron (Flv) proteins that act as “electron valves”, accepting excess electrons when the ETC is over-reduced (Mullineux, 2013). It was proposed that in wild type conditions, Flv2/4 accept electrons directly from $Q_B$, temporarily diverting them from the ETC and preventing back-reduction of $Q_A$ and thus preventing photooxidative damage (Zhang et al., 2012). It appears that Flv1/3 perform a similar function as Flv2/4, but instead act around PSI (Allahverdiyeva et al., 2013). Thus, the Flv electron reservoirs have the potential to prevent photoinhibition by preventing overreduction of the ETC (Bersanini et al., 2014).

Pulse-Amplitude-Modulation (PAM) fluorometry measures fluorescence from $Q_A$, and thus fails to differentiate the relative redox poise of all downstream components of the ETC, such as $Q_B$ and PQ (Campbell et al., 1998). Instead, to differentiate between $Q_B$ and PQ, exogenous quinones with varying redox potentials can be used to accept electrons from specific positions within the ETC. For example, it has recently been shown that the quinones DCBQ and PPBQ oxidized $Q_B$, while DMBQ accepts electrons directly from PQ thereby maintaining a relatively more reduced $Q_B$/PQ (Fu et al., 2017). Thus, differential gene expression in the presence of these inhibitors would suggest regulation via the redox state of $Q_B$. Furthermore, incubation of cyanobacteria with oxidized quinones has been shown to mimic the onset of darkness through an effect on the phosphorylation state of KaiC, linking ETC redox status with functioning of the circadian clock (Kim et al., 2012).

The ability of *Synechocystis* to utilize changes in the ETC redox poise, either at $Q_B$ or PQ, to perceive diverse abiotic stresses has recently been shown using ETC inhibitors and PAM fluorometry (Ritter *et al.,* 2019). It was proposed that the common effect of diverse environmental conditions on the redox poise of $Q_B$/PQ acts as a convergent sensing mechanism. This mechanism therefore functions in the absence of individual two-component signaling pathways specific for each stress. The data indicated that enhanced reduction of the ETC surrounding $Q_B$/PQ induced expression of the DEAD-box RNA helicase CrhR. However, the exact point of the ETC utilized for redox poise sensing and the presumed two-component signaling pathway leading to *crhR* induction were not identified.

Here a series of quinones that accept electrons from specific locations within the ETC were utilized to provide evidence that the redox poise of $Q_B$ enacts changes in *crhR* expression. Furthermore, a series of mutants in two-component signaling systems previously linked with redox signaling downstream of the ETC were analyzed to identify the two-component signaling system that conveys the $Q_B$ redox poise status. Although none of the tested HK or RR mutants fully eliminated stress induced CrhR expression, a decreased rate of CrhR accumulation in response to both temperature and salt stress suggested the Hik8/RpaA/RpaB pathway is involved. The partial effect of *rpaA* deletion is likely reflective of overlapping functionality with *rpaB* or redundant signaling through Hik8 via a yet to be identified RR. CrhR has been previously linked to regulating the

expression of various small regulatory RNAs (sRNAs) and stress responsive and photosynthetic genes (Georg et al., 2019). Thus, this study describes a mechanism by which photosynthetic homeostasis is maintained through environmental perception by alteration of the redox poise of $Q_B$ which is linked to changes in CrhR expression through the Hik8/RpaA/RpaB signaling pathway.

## 4.3 Materials and methods

*4.3.1 Strains and culture conditions*

The glucose tolerant strain of *Synechocystis* sp. PCC 6803 (Kujat and Owttrim, 2000) was maintained and grown on BG-11 medium at 30°C in continuous white light and aerated by shaking at 150 rpm and bubbling with humidified air as described previously (Owttrim, 2012). Experiments utilized cultures with chlorophyll *a* (Chl *a*) concentrations ranging from 5-7 µg/mL. Media used for growth of mutant cyanobacteria was supplemented with sodium thiosulfate (0.3%), tricine (10mM, pH 8.0). Selection was maintained in transformed and inactivation mutant strains using kanamycin (50 µg $ml^{-1}$, pJA2-Rre1*,* Δ*hik30/*Δ*rre33,* and Δ*hik34*), chloramphenicol (25 µg $ml^{-1}$, Δ*rpaA*) and chloramphenicol and spectinomycin (25 µg $ml^{-1}$, Δ*flv1*/3). The Δ*psbO,* Δ*psbH and* Δ*hik33* strains were grown in the presence of 10 mM glucose.

*4.3.2 DNA manipulation*

The ΔHLR1 site inactivation was constructed using the QuikChange mutagenesis protocol outlined in Xia and Xun (2017). Mutagenesis was performed on a plasmid derived from pMON 36456 (Qi et al., 2005), where the constitutive *nirA* promoter was replaced with the previously defined promoter and open reading frame of *crhR,* (Kujat and Owttrim, 2000) placed downstream of the *slr0082* reading frame and promoter. This construct is reffered to as "A1". Primers used for mutagenesis were: ΔHLR1_F: TCCCCTGTCTTTCCCCATAAAAAGGGTCTGACCACCGTTC, ΔHLR1_R: GGGGAAAGACAGGGGAACAGATTTACACCAGAAAACCTGAGG. Deletion of the HLR1 binding sequence was confirmed by DNA sequencing using the primer LPF73: GTATCAGATCGTTAATTCC. After verification by DNA sequencing, the ΔHLR1

plasmid was transformed into the Δ*crhR* strain lacking the entire *crhR* ORF (Tarassova et al., 2014), using triparental mating as previously described (Owttrim, 2012).

*4.3.3 Stress and inhibitor conditions*

Illuminated mid-log phase cultures were subjected to the following stress conditions for the indicated times: 20°C cold stress, 0.6 M NaCl, 20 µM $CoCl_2$, 40 µM 2,6-dimethoxy-1,4-benzoquinone (DMBQ), 40 µM phenyl-p-benzoquinone (PPBQ), 40 µM 2,6-dichloro-1,4-benzoquinone (DCBQ), 1 mM ferricyanide ($K_3[Fe(CN)_6]$). The quinones and ferricyanide were dissolved in water while the electron transport inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 2.5 µM) and dibromothymoquinone (DBMIB, 10 µM) were dissolved in DMSO as described previously (Kujat and Owttrim, 2000). DMSO (0.066%) was added to a control culture when DCMU and DBMIB were used.

*4.3.4 Protein abundance*

Wild type CrhR (55 kDa) abundance was determined by western blot analysis of soluble protein (20 ug) using anti-CrhR antibody (1:5,000) (Kujat and Owttrim, 2000) and ECL detection (ECL, Bio-Rad Clarity Western ECL Substrate, Mississauga, ON), as previously described (Owttrim, 2012). When shown, the abundance of Rps1 was used as a protein loading control using anti-*Escherichia coli* Rps1 (1:5,000) and ECL detection, as previously described (Rosana et al., 2012). Soluble protein was quantified using a Bradford assay with BSA as the standard.

## 4.4 Results:

In an effort to delineate the position of redox signaling within the cyanobacterial ETC, CrhR expression was analyzed in mutants disrupted at three major sites of signaling, *psbO, psbH* and *flv1/3* (Fig. 4.1). Normal induction of CrhR in response to temperature downshift in wild type cells is shown in Figure 4.1A. A basal level of CrhR is observed at 30ºC which increases to a maximum within 3 hours. Upon return to the normal growth temperature at 30ºC, proteolysis returns CrhR to basal levels within a similar time frame. In the absence of Flv1/3, which act to dissipate excess electron pressure around PSI, low temperature induction and temperature upshift repression of CrhR proceeded similarly to

wild type (Fig. 4.1B). *psbO* and *psbH* act within PSII, playing roles in water splitting and electron flow between $Q_A$ and $Q_B$ respectively (Komenda and Barber, 1995). As such, deletion of *psbH,* but not *psbO* has been reported to cause accumulation of photodamage at the $Q_B$ site under high light stress. Therefore, if $Q_B$ is responsible for enacting changes in CrhR gene expression, induction should be impeded in the *psbH* mutant strain. However, *psbO* and *psbH* both showed wild type expression of CrhR in response to temperature downshift and upon return to the normal growth temperature (Fig. 4.1C and D).

To further localize the position of ETC redox sensing responsible for induction of CrhR, the effect of exogenous quinones was analyzed. Both DCBQ and PPBQ have been shown to accept electrons directly from $Q_B$, while DMBQ accepts electrons from the PQ pool. At the normal growth temperature, both DCBQ and PPBQ caused minor induction of CrhR (Fig. 4.2A and B), while DMBQ caused a significant decrease in abundance (Fig. 4.2C). Upon temperature downshift to 20ºC, induction of CrhR occurred normally in the presence of the three quinones (Fig. 4.2A-C). In order to prevent back reduction of both $Q_B$ and PQ and to keep the quinones in an oxidized state, ferricyanide was added in combination with the exogenous quinones. Under these conditions, induction of CrhR at 20ºC was inhibited by DCBQ and PPBQ which accept electrons from $Q_B$, but not DBMQ which accepts electrons from PQ and therefore allows $Q_B$ to remain relatively reduced. (Fig. 4.2D-F). Treatment with ferricyanide alone did not significantly affect either induction or repression of CrhR accumulation, suggesting that the results originate from quinone oxidation of the ETC (Fig. 4.2G).

It has recently been shown that unlike the response in higher plants, cyanobacterial state transitions are not controlled by Ser/Thr Kinase phosphorylation, suggesting a HK two-component signaling pathway activated by enhanced reduction of the ETC is associated with this response (Calzadilla *et al.,* 2019). A number of two-component signaling systems have been shown to be responsive to the redox poise of the ETC and/or facilitate gene regulation in response to diverse abiotic stresses. In order to determine if a similar system was responsible for redox regulation of CrhR, protein abundance was determined in a series of HKs and RRs known to be redox-responsive (Fig. 4.3). Since *rre1* is an essential gene in *Synechocystis*, we analyzed the effect of Rre1 overexpression on CrhR accumulation. Both induction in response to temperature downshift and proteolysis

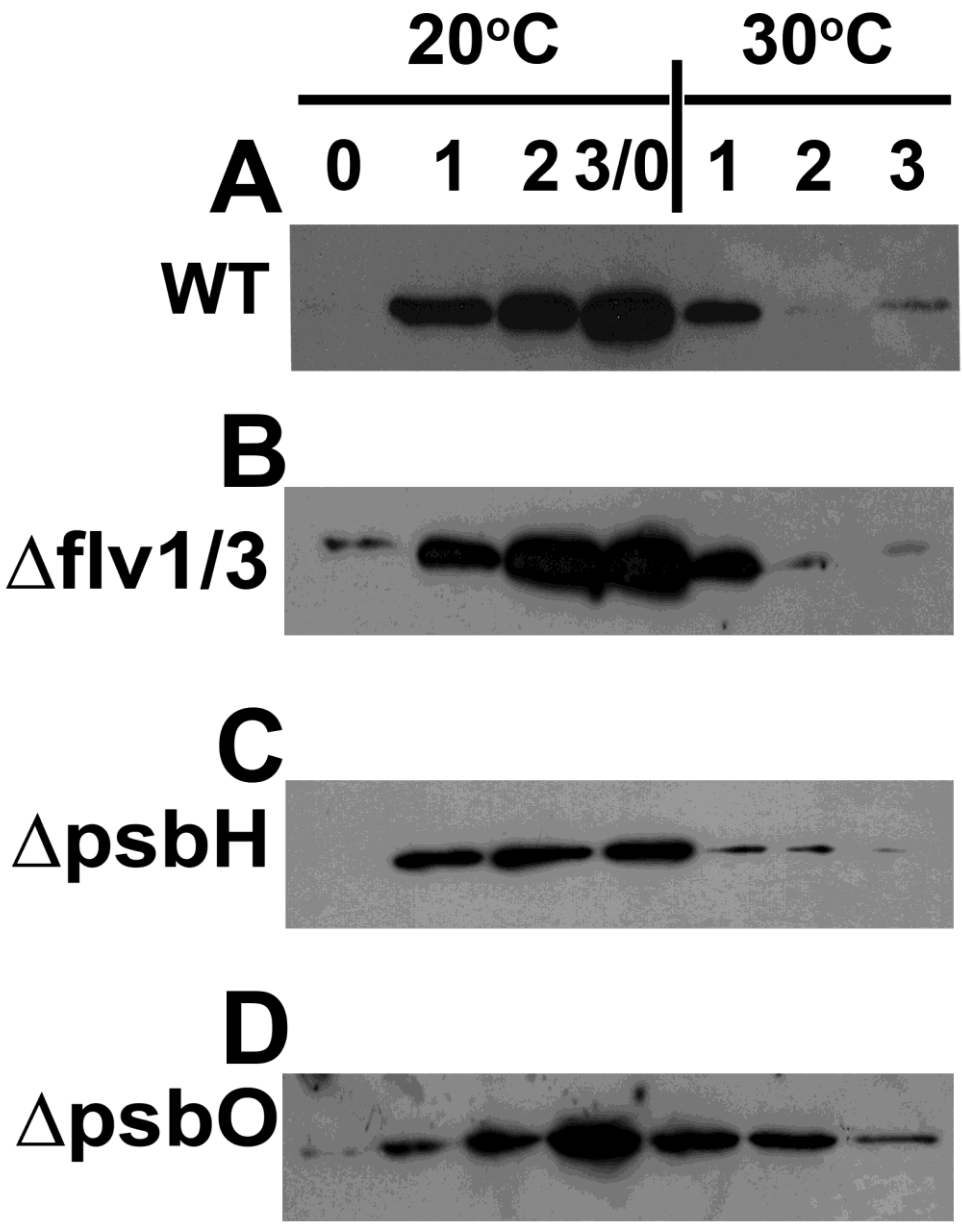



**Fig 4.1 – Effect of electron transport chain mutants on CrhR expression.**
*Synechocystis* cells were grown to mid-log phase at 30ºC before shifting to 20ºC for 3 h. Cells were then returned to 30ºC 3 h additional hours. Cultures were sampled immediately before temperature downshift and every hour subsequently. Both wild type (WT) and various mutant *Synechocystis* cultures were tested. (A) WT. (B) Δ*flv1/3* (C) Δ*psbH* (D) Δ*psbO.*

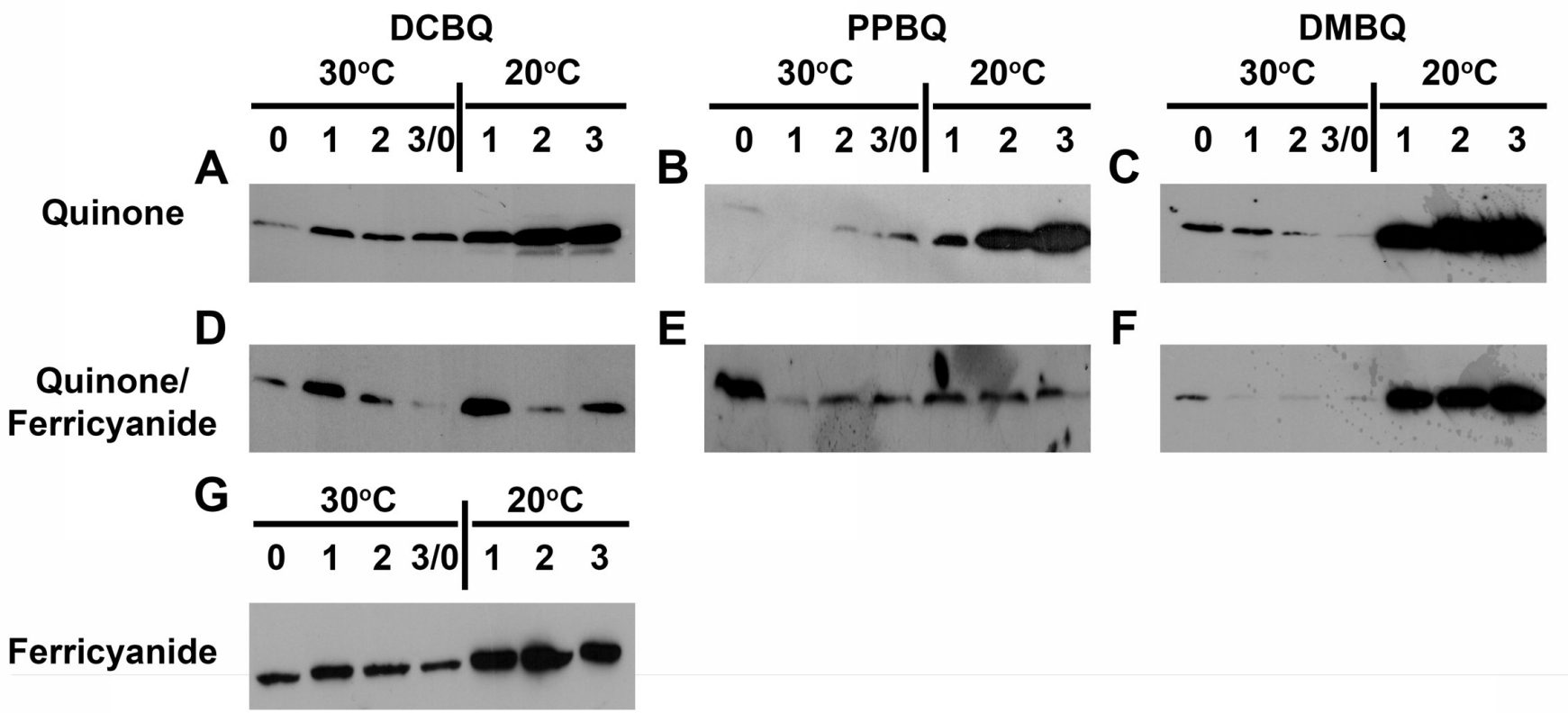



**Fig 4.2 – Provision of exogenous quinones in the presence and absence of ferricyanide affects expression of CrhR.** *Synechocystis* cells were grown to mid-log phase at 30ºC before exogenous quinones predicted to oxidize either $Q_B$ (DCBQ/PPBQ) or PQ (DMBQ) were added. Where indicated, ferricyanide was also included both alone and with exogenous quinones to act as a control and to maintain quinones in an oxidized state, respectively. Cells were incubated at 30ºC for 3 h before shifting to 20ºC for an additional 3 h. Cells were sampled immediately before addition of exogenous quinones/ferricyanide and sampled every hour subsequently (A-C). Addition of individual quinones in the absence of Ferricyanide. (A) DCBQ (B) PPBQ (C) DMBQ. (D-F) Addition of individual quinones in the presence of ferricyanide. (D) DCBQ (E) PPBQ (F) DMBQ. (G) Addition of ferricyanide in the absence of exogenous quinones.

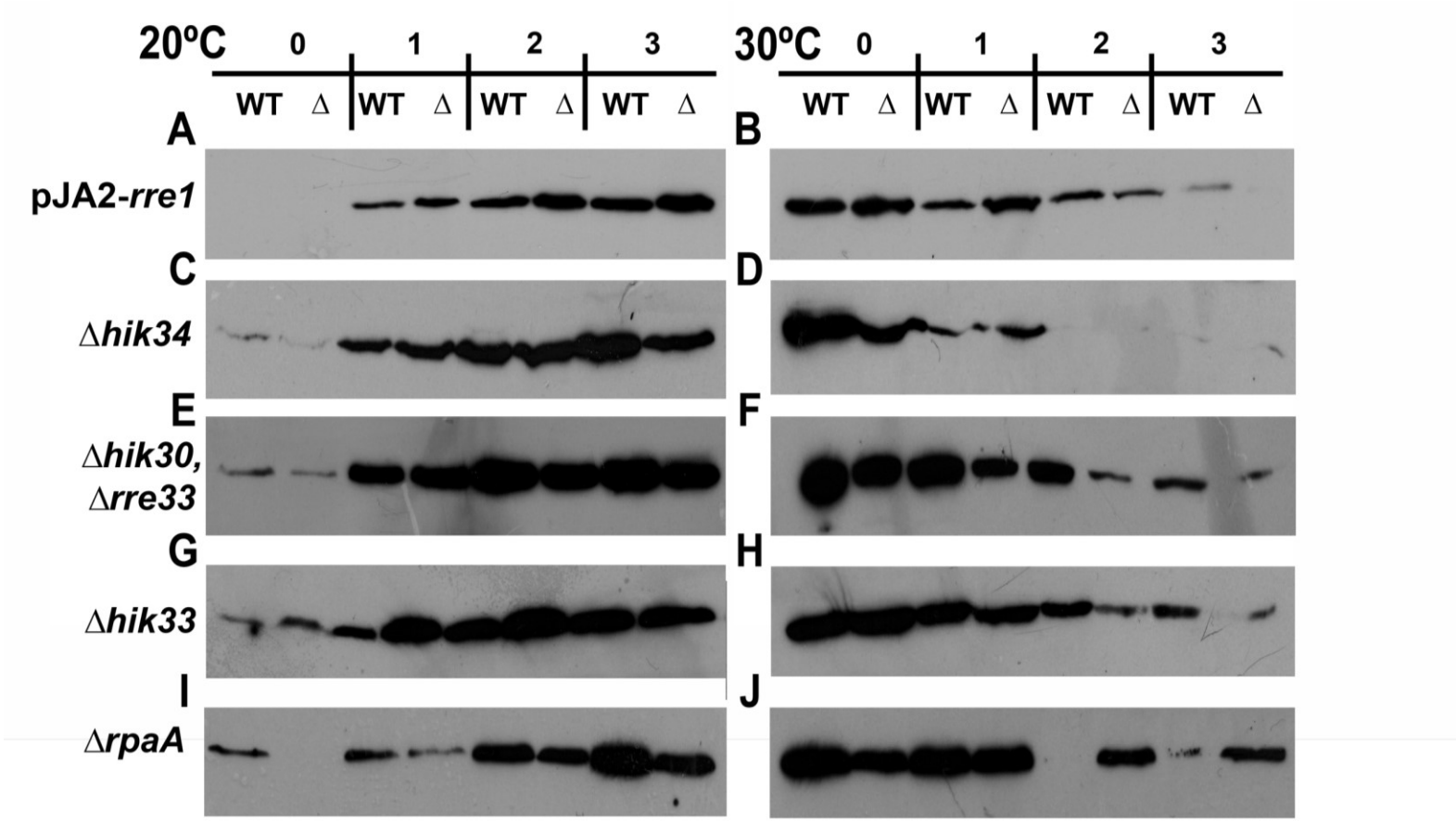



**Fig 4.3 – Temperature mediated induction and repression of CrhR in various two-component signaling mutants.** *Synechocystis* cells were grown to mid-log phase at 30ºC before being moved to 20ºC for 3 h. Cells were then returned to 30ºC 3 h additional hours. Cultures were sampled immediately before temperature downshift and every hour after that. WT and various mutant *Synechocystis* cultures were sampled simultaneously and are displayed side by side. (A-B) pJA2-Rre1 (C-D) Δ*hik30,* Δ*rre33* (E-F) Δ*hik34* (G-H) Δ*hik33* (I-J) Δ*rpaA.*

upon return to normal growth temperature proceeded to similar levels at similar rates in both the wild type and cells overexpressing Rre1 (pJA2-Rre1) (Fig 4.3A-B). Evidence that Hik34-Rre1 are not associated with *crhR* regulation was also provided by the observation that induction also occurred at 20ºC in the absence of Hik34, which has previously been shown to phosphorylate Rre1 in response to heat shock (Kobayashi et al., 2017) (Fig. 4.3C-D). Similarly, CrhR induction and proteolysis in response to temperature downshift in the absence of the redox responsive two-component signaling pathway in the double mutant, Δ*hik30,* Δ*rre33*, was also not affected (Fig. 4.3E-F). Direct involvement of Hik33 was also discounted by the normal temperature regulated induction and repression of CrhR observed in a Δ*hik33* strain (Fig. 4.3G-H). In addition, alteration of membrane fluidity using benzyl alcohol, a treatment known to activate Hik33 (Mironov et al, 2012, Mironov et al, 2017), also failed to alter CrhR induction in response to temperature downshift (Fig 4.4A).

Similarly, provision with benzyl alcohol did not relieve inhibition of DCMU, confirming CrhR redox regulation proceeds independently of membrane fluidity (Fig 4.4B). However, deletion of the Hik33 cognate RR, RpaA, caused a substantial decrease in the rate of both CrhR induction and proteolysis (Fig. 4.3I-J).

We then determined CrhR induction in response to various stresses, which have previously been demonstrated as capable of activating each two-component signaling system. *crhR* has been demonstrated to be responsive to all diverse abiotic stresses tested here (Ritter et al., 2019). The ability of NaCl and $CoCl_2$ to induce CrhR with similar kinetics to cold shock has been previously demonstrated (Ritter et al., 2019). The Hik34 and Hik30/Rre33 two-component signaling systems have been previously demonstrated to facilitate gene regulation in response to NaCl and $CoCl_2$, respectively. Induction of CrhR continued to increase linearly in response to extended exposure to NaCl in the Δ*hik34* strain (Fig. 4.5C), indicating that Hik34 is not associated with $Na^+$ signaling to CrhR expression. Similarly, cobalt stress signaling to CrhR did not occur through Δ*hik30*-Δ*rre33,* as expression peaked followed by a slow decline in Δ*hik30*-Δ*rre33* cells after addition of $CoCl_2$ (Fig. 4.5D), identical to the transient induction pattern previously observed in wild type cells (Ritter et al., 2019). However, as observed with temperature

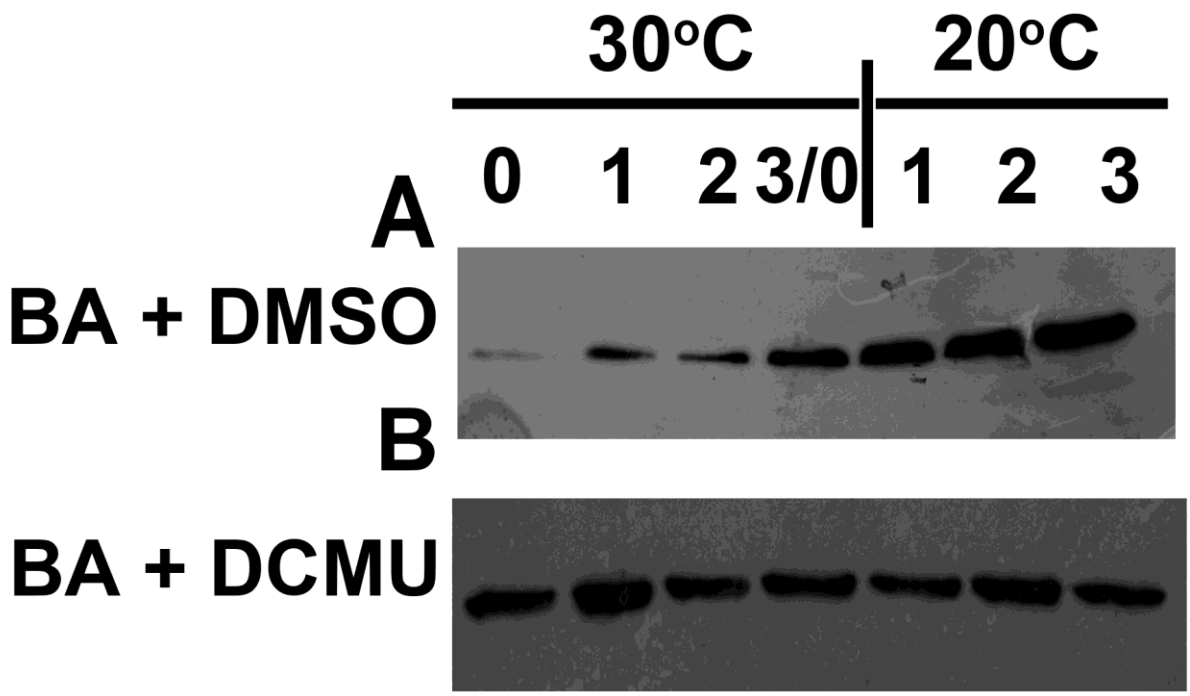



**Fig 4.4. Effect of benzyl alcohol on temperature and redox control of CrhR expression.** *Synechocystis* cells were grown to mid-log phase at 30ºC before the benzyl alcohol was added. Where indicated, either a (A) DMSO solvent control or (B) DCMU was also provided. Cells were then kept at 30ºC for 3 h before shifting to 20ºC for an additional 3 h. Cells were sampled immediately before addition of benzyl alcohol sampled every hour after that.

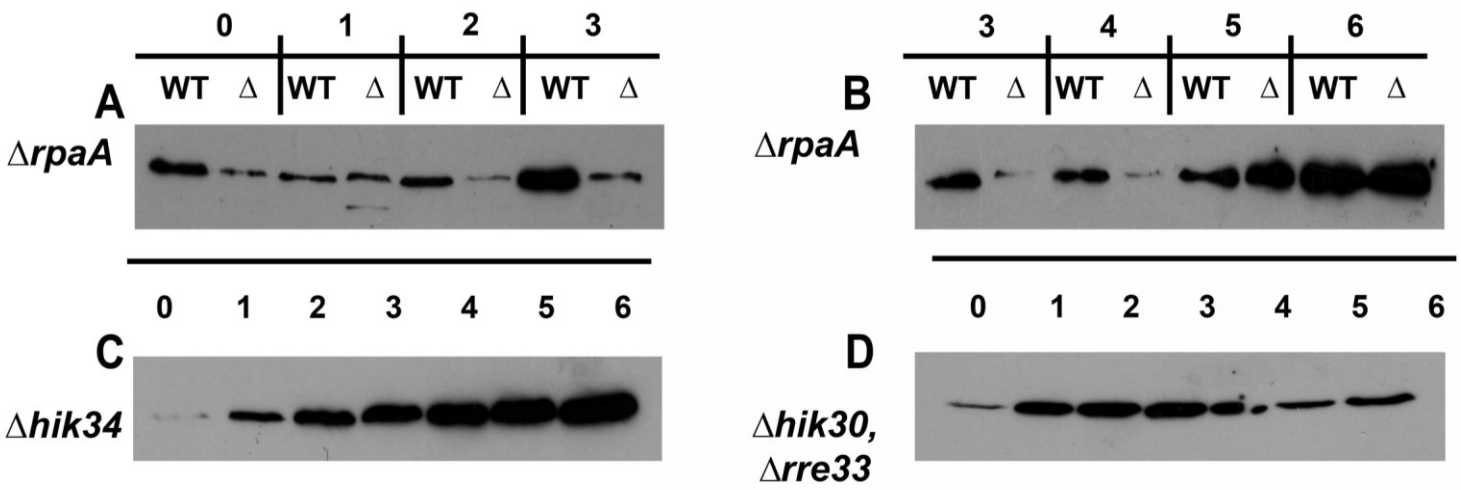



**Fig 4.5 – Diverse abiotic stress mediated induction and repression of CrhR in various two-component signaling mutants.** *Synechocystis* cells were grown to mid-log phase at 30ºC before being exposed to either NaCl (A-C) or $CoCl_2$ (D). Cultures were sampled immediately before application of abiotic stress and every hour after that for 6 h. (A-B) WT and Δ*rpaA Synechocystis* cultures were sampled simultaneously and are displayed side by side (C) Δ*hik34* (D) Δ*hik30,*Δ*rre30.*

stress, deletion of RpaA, caused a significant delay in NaCl induction of CrhR accumulation (Fig. 4.5AB).

To further investigate the potential contribution of RpaA to the transcriptional regulation of CrhR expression, mutagenesis of the predicted HLR1 RpaA binding site upstream of CrhR was performed. Mutagenesis of this site without changing the length or potential secondary structure of this region did not prevent induction or repression of CrhR in response to either temperature when compared to expression from the A1 plasmid with an unaltered HLR1 site (Fig. 4.6A and B). These results suggested that the HLR1 promoter element is not required for expression of CrhR.

## 4.5 Discussion

Utilization of the electron transport inhibitors DCMU and DBMIB in conjunction with genetic analysis has been interpreted to indicate that the redox poise of PQ regulates expression of multiple genes in cyanobacteria and higher plants (Zito et al., 1999; Kujat and Owttrim 2000; Alfonso et al., 2000; Ibrahim et al., 2016). One of these genes is the RNA helicase *crhR,* which is induced by diverse abiotic stress and plays a role in maintenance of photosynthesis in response to changing environmental conditions (Georg et al, 2019; Ritter et al, 2019). Here we provide evidence that sensing of the $Q_B$ redox poise elicits gene regulation as quinones that accepted electrons from $Q_B$, but not PQ, inhibited abiotic stress induction of CrhR. Analysis of HK-RR mutant strains suggests that *rpaA* plays a role in linking the redox poise of $Q_B$ with CrhR expression. *rpaA* activation is expected to proceed through Hik8, a key output of the KaiABC circadian oscillator in *Synechocystis (*Osanai et al., 2015) (Fig. 4.7).

Limited evidence has been presented suggesting that $Q_B$ is the ETC component whose redox status is sensed. Komenda and Barber (1995) reported differential regulation of D1 gene expression in response to deletion of the PSII components *psbO* and *psbH*. Since these mutants have previously identified to have divergent effects on the redox status of $Q_B$, the results were interpreted to indicate $Q_B$ as the sensing site. Unfortunately, CrhR stress regulation was not affected by mutation of either *psbO* or *psbH*, previously identified to have divergent effects on the redox status of $Q_B$. The reason for this observation is not immediately evident however we

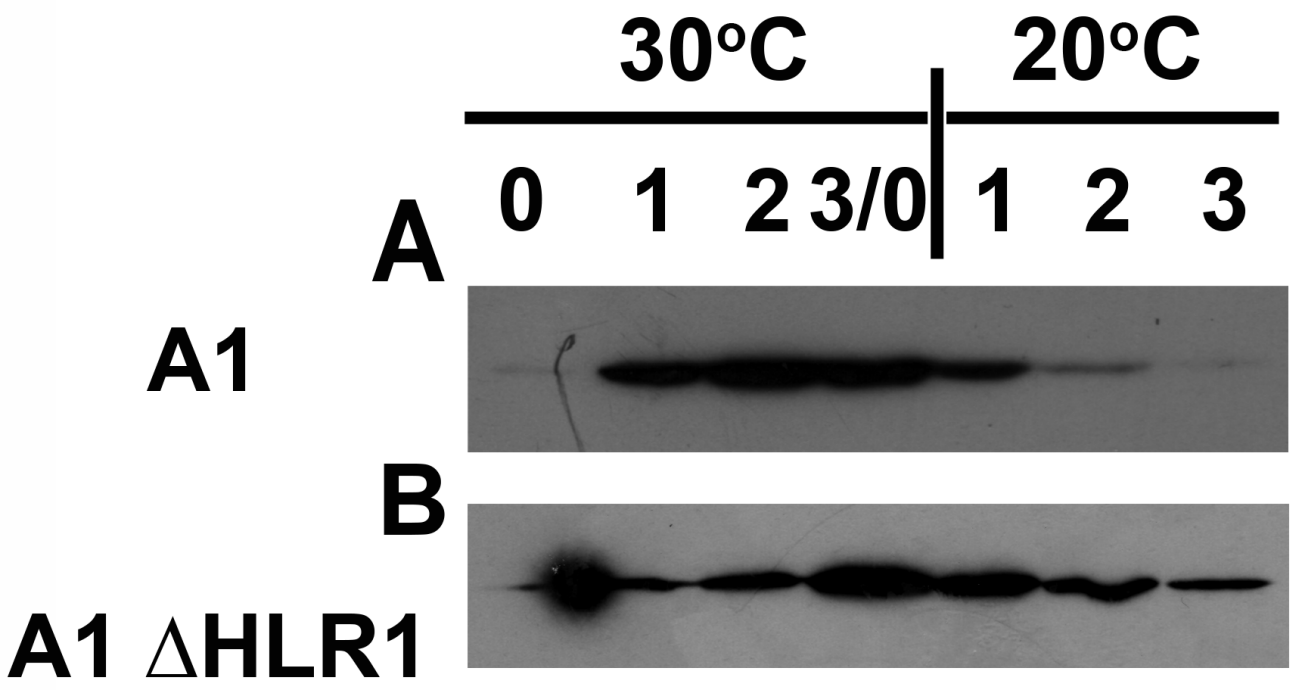



**Fig 4.6 – Effect of temperature and salt on CrhR expression in the absence of a predicted upstream HLR site.**
Δ*crhR* cells were transformed with either the (A) A1 plasmid or the (B) ΔHLRA1 plasmid which lacks the HLR1 site upstream of *crhR*. Both cultures were grown until log phase at 30ºC before being exposed to cold stress (20ºC). Cultures were sampled immediately before application of abiotic stress and every hour after that for 6 h. Cultures initially placed at 20ºC were returned to 30ºC after 3 h. (A) Response of CrhR expressed from the A1 plasmid in response to temperature. (B) Response of CrhR expressed from the ΔHLR1A1 plasmid in response to temperature.

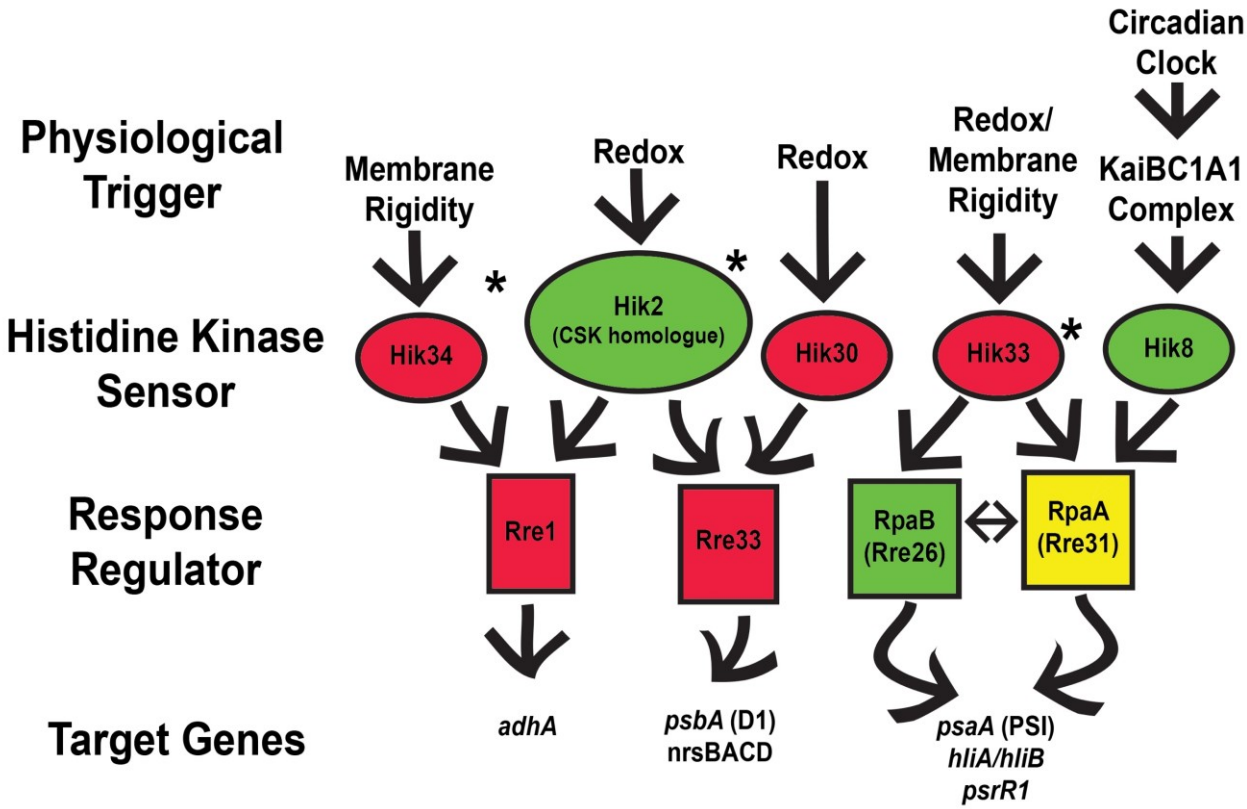



**Figure 4.7 – Overview of potential two-component signaling mechanisms responsible for induction of CrhR.**
Numerous two-component signaling systems have been suggested to respond to diverse abiotic stresses in *Synechocystis*. Physiological triggers which facilitate downstream changes in gene regulation include membrane rigidity, the redox poise of the electron transport chain and circadian rhythm. HKs which are activated by these triggers are displayed. Interactions between a single HK with multiple downstream RRs is common, as is the case for *hik2* and *hik33*. Similarly, RRs, such as *rre1, rre33* and *rpaA* can be activated by multiple HKs. In addition, RRs including *rpaA* and *rpaB* have also been shown to influence each other's[wording] activity at the protein level. Red designates candidates directly tested and confirmed not to have an effect on CrhR expression in response to diverse abiotic stresses. Green designates essential genes which could not be investigated directly, but still may contribute to regulation of CrhR. Yellow indicates RpaA which appears to be involved in regulation of CrhR expression, either directly or indirectly. Select examples of downstream genes responsive to each RR are also provided.

propose that the effect may have resulted from the different abiotic stresses employed, high light vs cold shock, as high light would be expected to cause enhanced levels of photodamage at $Q_B$ compared to cold stress (Ermakova-Gerdes and Vermaas, 1998; Guskov *et al.,* 2009; Luo *et al.,* 2014; Fu *et al.,* 2017). Thus, potentially the functionality/abundance of $Q_B$ would be relatively unaffected in the *psbH* in response to low temperature thereby resulting in a reduced effect on $Q_B$ oxidation and thus no effect on CrhR expression. A potential contribution of the redox status of *flv1/3* or *flv2/4*, known to be associated with PSI and PSII activity, respectively (Zheng et al., 2012, Allahverdiyeva et al., 2013), was not observed as CrhR expression was not altered in these mutants. The *flv* mutant results also provided additional evidence supporting the conclusion that enhanced ROS levels induced by over-reduction of the ETC was not responsible for CrhR regulation, similar to those previously reported (Ritter et al. 2019).

When applied under oxidizing conditions, quinones capable of accepting electrons from $Q_B$, but not PQ, prevented induction of CrhR. This effect required the presence of ferricyanide as an electron sink, confirming that the observed effects result from altered redox signaling and that $Q_B$ and not PQ facilitates redox signaling. This is further supported by mutations around the $Q_C$ hydrophobic pocket, which is proposed to contain $Q_B$, causing defects in state transition and photosynthetic growth rate (Guskov et al., 2009; Huang et al., 2016; Huang et al., 2018; Calzadilla et al., 2019). However, existence of this site is controversial (Umena et al., 2011). Nevertheless, utilization of $Q_B$ could be a more obvious candidate to represent the overall redox status of the ETC than PQ, which exists as a freely diffusible pool within the thylakoid membrane (Calzadilla et al., 2019).

The range of HK-RR systems that have been associated with various aspects of redox and/or abiotic stress sensing in cyanobacteria (Fig. 4.7) indicates that not all of the signal transduction occurs through a common two-component signaling pathway. For example, Hik2 is responsive to the redox poise in the vicinity of the PQ pool, involving interactions with the RRs, Rre1 and Rre33 (Ibrahim et al., 2016). Here, systematic analysis of HK-RR systems known to be associated with response to abiotic stress and/or redox poise only revealed involvement of RpaA in regulation of CrhR expression. Deletion of RpaA, which acts downstream as a response regulator of Hik33, exhibited altered expression of CrhR in response to treatment with NaCl and cold shock. Hik33 is proposed

to respond to the PQ pool in a membrane fluidity dependent fashion (Maksimov et al., 2017). However, CrhR expression was unaffected by a combination of temperature change in the absence of Hik33, and alteration of membrane fluidity through addition of benzyl alcohol. This was expected, as CrhR has previously been suggested to proceed independently of Hik33 (Suzuki et al., 2001; Mironov et al., 2012; Mironov et al., 2014; Ge et al., 2017; Maksimov et al., 2017)

As deletion of Hik33 did not affect expression of CrhR, this suggests that RpaA regulation of CrhR expression involves $Q_B$ redox sensing by Hik8. Hik8 interacts with the central circadian oscillator KaiABC which has been demonstrated to enact changes in gene regulation in response to ETC redox status primarily through RpaA (Takai et al., 2006; Kuwahara et al., 2015). *Synechocystis* lacking functional *rpaA* display decreased viability when grown under light - dark photoperiods, suggesting a conserved role in coordinating gene expression under these conditions (Köbler et al., 2018). In association with light – dark regulation, *rpaA* has previously been described as the primary output of the circadian clock in *Synechococcus elongatus* (Takai et al., 2006; Markson et al., 2013; Fleming and O'Shea, 2018).

RpaA has previously been shown to facilitate gene regulation in response to high light conditions through interaction with the conserved promoter element HLR1 in concert with RpaB (Takahashi et al., 2010; Espinosa et al., 2015). Ablation of the conserved repeats of the HLR1 site has previously been demonstrated to abolish RpaB binding within the shared promoter of the *psaAB* genes (Takahashi et al., 2010). A predicted HLR1 site was recently identified upstream of the *crhR* promoter via computational biology (Riedeger et al. 2019). Both RpaA and RpaB have been shown to bind to HLR1, facilitating changes in expression of genes such as the high-light-inducible protein *hliB*, the PSI genes *psaAB* and PsrR1, a sRNA which plays a role in regulation of photosystem stoichiometry (Kappell and Waasbergen, 2007; Seino et al., 2009; Kadowaki et al., 2016). Unexpectedly, disruption of the potential HLR1 site did not alter induction or proteolysis of CrhR in response to temperature change. These results could potentially involve auto-regulatory interactions between RpaA and RpaB (Fig. 4.7). RpaB has been demonstrated to compensate for RpaA deletion by binding to HLR1 sites and RpaB overexpression has been shown to inhibit RpaA phosphorylation (Espinosa et al., 2015). This demonstrates

that RpaA and RpaB regulation is partially redundant at the promoter level and involves interactions at the protein level. Thus, interaction with RpaB may contribute to the incomplete effect of RpaA deletion on CrhR induction and repression.

Acting downstream of Hik8/RpaA, CrhR could facilitate post-translational regulation of circadian gene expression through RNA helicase activity, allowing for rapid changes in gene expression at light - dark transitions. Unfortunately, the direct involvement of Hik8-RpaB in CrhR regulation cannot genetically assessed since both genes are essential (Ashby and Mullineux, 1999; Puthiyaveetil et al., 2008).

In summary, utilization of alternative quinones with varying redox activities provided evidence that the redox status of $Q_B$ and not PQ is the site responsible for induction of CrhR. Quinones have also been shown to be required for expression of *ArcA*/*B,* a two-component signaling system in *E. coli* which responds to diverse abiotic stress, suggesting similar mechanisms may be widespread in bacteria (van Bielen and Hellingwerf, 2016). In order to identify the HK-RR pathway responsible for linking the ETC with CrhR expression, analysis of mutants for all non-essential two-component systems demonstrated to be influenced by redox was performed. Only deletion of RpaA resulted in altered CrhR expression in response to application of abiotic stress. Since Hik33 was shown to not be required for CrhR expression, this suggested that RpaA is activated by Hik8. Hik8 has been characterized as interacting with the KaiABC complex, providing a link between *crhR* expression and the circadian clock in *Synechocystis.* The inability of *rpaA* deletion to completely prevent CrhR induction suggests compensation by an associated RR, potentially RpaB or a yet to be identified RR downstream of Hik8. Further confirmation of Hik8-RpaB involvement with CrhR expression requires antisense knockdown of one or both genes. How redox regulation in response to $Q_B$ would be sensed by Hik8 remains unclear.-The complex orchestration of interacting two-component signaling networks which facilitate redox responsive gene regulation in *Synechocystis* demonstrate the crucial role of ETC derived signals for adaptation to changing environmental conditions.

## Acknowledgements

I am deeply thankful to the cyanobacterial community at large for their willingness to provide mutant strains for this project: Thanks to Dr. Luis Lopez Maury (Δ*hik30*/Δ*rre33*), Dr. Elton Hudson (Δ*hik34* and pJA2-*rre1*), Dr. Eva-Marie Aro (Δ*flv1* and Δ*flv3*), Dr. Annegret Wilde (Δ*rpaA*), and Dr. Qingfang He (Δ*hik33*) for their generous provision of mutants, Drs. Danuta Chamot and Laura Patterson-Fortin for cloning of the A1 plasmid and Oxana Tarassova for generation of the Δ*crhR* strain. Special thanks to Dr. Josef Komenda for providing both the Δ*psbO* and Δ*psbH* strains and stimulating discussion on the potential for $Q_B$-mediated regulation in cyanobacteria.

## 4.5 References

Alfonso A, Perewoska I, Kirilovsky D. 2000. Redox Control of *psbA* Gene Expression in the Cyanobacterium *Synechocystis* PCC 6803. Involvement of the Cytochrome $b_6f$ Complex. Plant Physiology 122, 505-515.

Allahverdiyeva Y, Mustila H, Ermakova M, Bersanini L, Richaud P, Ajlani G. 2013. Flavodiiron proteins Flv1 and Flv3 enable cyanobacterial growth and photosynthesis under fluctuating light. Journal of Bacteriology 110, 4111–4116.

Ashby MK, Houmard J. 2006. Cyanobacterial two-component proteins: structure, diversity, distribution, and evolution. Microbiology and Molecular Biology Reviews 70, 472–509.

Ashby MK, Mullineaux CW. 1999. Cyanobacterial *ycf27* gene products regulate energy transfer from phycobilisomes to photosystems I and II. FEMS Microbiology Letters 181, 253–260.

Bersanini L, Battchikova N, Jokel M, Rehman A, Vass I, Allahverdiyeva Y. 2014. Flavodiiron protein Flv2/Flv4-related photoprotective mechanism dissipates excitation pressure of PSII in cooperation with phycobilisomes in cyanobacteria. Plant Physiology 164, 805–818.

Calzadilla PI, Zhan J, Sétif P, Lemaire C, Solymosi D, Battchikova N, Wang Q, Kirilovsky D. 2019. The cytochrome $b_6f$ complex is not involved in cyanobacterial state transitions. Plant Cell 31, 911–931.

Campbell D, Hurry V, Clarke AK, Gustafsson P, Oquist G. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiology and Molecular Biology Reviews 62, 667–683.

Ermakova-Gerdes S, Vermaas W. 1998. Mobility of the primary electron-accepting plastoquinone QA of photosystem II in a *Synechocystis* sp. PCC 6803 strain carrying mutations in the D2 protein. Biochemistry 37, 11569-11578.

Espinosa, J, Boyd JS, Cantos R, Salinas P, Golden SS, Contreras A. 2015. Crosstalk and regulatory interactions between the essential response regulator RpaB and cyanobacterial circadian clock output. Proceedings of the National Academy of Sciences of the United States of America 112, 7.

Fleming KE, O'Shea EK. 2018. An RpaA-Dependent Sigma Factor Cascade Sets the Timing of Circadian Transcriptional Rhythms in *Synechococcus elongatus*. Cell Reports 25, 2937–2945

Fu HY, Picot D, Choquet Y, Longatte G, Sayegh A, Delacotte J, Wollman FA. 2017. Redesigning the $Q_A$ binding site of Photosystem II allows reduction of exogenous quinones. Nature Communications 8, 15274.

Ge H. 2017. Translating Divergent Environmental Stresses into a Common Proteome Response through the Histidine Kinase 33 (Hik33) in a Model Cyanobacterium. 2017. Journal of Proteomics 16, 1258–1274.

Georg J, Rosana ARR, Chamot D, Migur A, Hess WR, Owttrim GW. 2019. Inactivation of the RNA helicase CrhR impacts a specific subset of the transcriptome in the cyanobacterium *Synechocystis* sp. PCC 6803. RNA Biology 16, 1205–1214.

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A, Saenger W. 2009. Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. Nature Structural & Molecular Biology 16, 334–342.

Hanaoka M, K Tanaka. 2008. Dynamics of RpaB-promoter interaction during high light stress, revealed by chromatin immunoprecipitation (ChIP) analysis in *Synechococcus elongatus* PCC 7942. Plant Journal 56, 327–335.

Ibrahim IM, Puthiyaveetil S, Allen JF. 2016. A two-component regulatory system in transcriptional control of photosystem stoichiometry: Redox-dependent and sodium ion-dependent phosphoryl transfer from cyanobacterial histidine kinase Hik2 to response regulators Rre1 and RppA. Frontiers in Plant Science 12, 196–212.

Iijima H, Shirai T, Okamoto M, Kondo A, Hirai MY, Osanai T. 2015. Changes in primary metabolism under light and dark conditions in response to overproduction of a response regulator RpaA in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, Frontiers in Microbiology 6, 888.

Joshua S, Mullineaux CW. 2004. Phycobilisome diffusion is required for light-state transitions in cyanobacteria. Plant Physiolology 135, 2112–2119.

Kappell AD, van Waasbergen LG. 2007. The response regulator RpaB binds the high light regulatory 1 sequence upstream of the high-light-inducible *hliB* gene from the cyanobacterium *Synechocystis* PCC 6803. Archive of Microbiology 187, 337–342.

Kadowaki T, Nagayama R, Georg J, Nishiyama Y, Wilde A, Hess WR, Hihara Y. 2016. A Feed-Forward Loop Consisting of the Response Regulator RpaB and the Small RNA PsrR1 Controls Light Acclimation of Photosystem I Gene Expression in the Cyanobacterium *Synechocystis* sp. PCC 6803. Plant and Cell Physiology, 57, 813–823.

Kim YI, Vinyard DJ, Ananyev GM, Dismukes GC, Golden SS. 2012. Oxidized quinones signal onset of darkness directly to the cyanobacterial circadian oscillator. Proceedings of the National Academy of Sciences of the United States of America USA 109, 17765–17769.

Köbler C, Schultz SJ, Kopp D, Voigt K, Wilde A. 2018. The role of the *Synechocystis* sp. PCC 6803 homolog of the circadian clock output regulator RpaA in day- night transitions. Molecular Microbiology 110, 847–861.

Komenda J, Barber J. 1995. Comparison of psbO and psbH deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the QB site and dependent on protein synthesis. Biochemistry, 34, 9625–9631.

Kujat SL, Owttrim GW. 2000. Redox-Regulated RNA Helicase Expression. Plant Physiology 124, 703-714.

Kuwahara A, Arisaka S, Takeya M, Iijima H, Hirai MY, Osanai T. 2015. Modification of photosynthetic electron transport and amino acid levels by overexpression of a circadian-related histidine kinase *hik8* in *Synechocystis* sp. PCC 6803. Frontiers in Microbiology 6, 637–9.

Li H, Sherman LA. 2000. A redox-responsive regulator of photosynthesis gene expression in the cyanobacterium *Synechocystis* sp. Strain PCC 6803. Journal of Bacteriology 182, 4268–4277.

Luo H, Jackson SA, Fagerlund RD, Summerfield TC, Eaton-Rye JJ. 2014. The importance of the hydrophilic region of PsbL for the plastoquinone electron acceptor complex of Photosystem II. Biochimica et Biophysica Actaf-Bioenergetics, 1837, 1435-1446.

Maksimov EG, Mironov KS, Trofimova MA, Nechaeva NL, Todorenko DA, Klementiev KE, Tsoraev GV, Tyutyaev EV, Zorina AA, Feduraev PV, Allakhverdiev SI, Paschenko VZ, Los DA. 2017. Membrane fluidity controls redox-regulated cold stress responses in cyanobacteria. Photosynthesis Research 133, 215–223.

Mao M. 2002. The redox state of plastoquinone pool regulates state transitions via cytochrome b. FEBS Letters. 519, 82–86.

Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N. 2003. Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis* sp. PCC 6803. Proceedings of the National Academy of Sciences of the United States of America USA 100, 9061–9066.

Markson JS, Piechura JR, Puszynska AM, O'Shea EK. 2013. Circadian Control of Global Gene Expression by the Cyanobacterial Master Regulator RpaA. Cell 155, 1396–1408.

Mikami K, Kanesaki Y, Suzuki I, Murata N. 2002. The histidine kinase Hik33 perceives osmotic stress and cold stress in *Synechocystis* sp PCC 6803. Molecular Microbiology 46, 905–915.

Mizuno T, Kaneko T, Tabata S. 1996. Compilation of All Genes Encoding Bacterial Two-component Signal Transducers in the Genome of the Cyanobacterium, *Synechocystis* sp. Strain PCC 6803. DNA Research 3, 407-414.

Mironov KS, Sidorov RA, Trofimova MS, Bedbenov VS, Tsydendambaev VD, Allakhverdiev SI., Los DA. 2012. Light-dependent cold-induced fatty acid unsaturation, changes in membrane fluidity, and alterations in gene expression in *Synechocystis*. Biochimica et Biophysica Acta 1817, 1352–1359.

Mironov KS, Sidorov RA, Kreslavski VD, Bedbenov VS, Tsydendambaev VD, and Los DA. 2014. Cold-induced gene expression and ω(3) fatty acid unsaturation is controlled by red light in *Synechocystis*. Journal of Photochemistry and Photobiology 137, 84–88.

Mullineaux CW. 2013. Co-existence of photosynthetic and respiratory activities in cyanobacterial thylakoid membranes. Biochimica et Biophysica Acta 1837, 503–511.

Osanai T, Shirai T, Iijima H, Kuwahara A, Suzuki I, Kondo A, Hirai MY. 2015. Alteration of cyanobacterial sugar and amino acid metabolism by overexpression hik8, encoding a KaiC-associated histidine kinase. Environmental Microbiology 17, 2430–2440.

Owttrim, G.W. 2012. RNA helicases in cyanobacteria: biochemical and molecular approaches. Methods in Enzymology 511, 385–403.

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I, et al. 2004. Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. Journal of Biological Chemistry 279, 53078–53086.

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF. 2008. The ancestral symbiont sensor kinase CSK

links photosynthesis with gene expression in chloroplasts. Proceedings of the National Academy of Sciences of the United States of America USA 105, 10061–10066.

Qi Q, Hao M, Ng WO, Slater SC, Baszis SR, Weiss JD, Valentin HE. 2005. Application of the *Synechococcus* nirA Promoter to Establish an Inducible Expression System for Engineering the *Synechocystis* Tocopherol Pathway. Applied and Environmental Microbiology 71, 5678–5684.

Riediger M. 2019. Biocomputational Analyses and Experimental Validation Identify the Regulon Controlled by the Redox- Responsive Transcription Factor RpaB. 2019. iScience 15, 316–331.

Ritter SPA, Lewis AC, Vincent S., Lo LL, Cunha APA, Chamot D, Ensminger I, Espie GS. Owttrim GW. 2019. Evidence for convergent sensing of multiple abiotic stresses in cyanobacteria. Biochemica et Biophysica Acta. 1864, 129462.

Rosana AR, Ventakesh M, Chamot D, Patterson-Fortin LM, Tarassova O, Espie GS, Owttrim GW. 2012b. Inactivation of a low temperature-induced RNA helicase in *Synechocystis* sp. PCC 6803: physiological and morphological consequences. Plant and Cell Physiology 53, 646–658.

Satoh K, Koike H, Ichimura T, Katoh S. 1992. Binding affinities of benzoquinones to the QB site of photosystem II in *Synechococcus* oxygen-evolving preparation. Biochimica et Biophysica Acta 1102, 5-52

Schuurmans RM, Matthijs JC, Hellingwerf KJ. 2017. Transition from exponential to linear photoautotrophic growth changes the physiology of *Synechocystis* sp. PCC 6803. Photosynthesis Research 132, 69–82.

Seino Y, Takahashi T, Hihara, Y. 2009. The Response Regulator RpaB Binds to the Upstream Element of Photosystem I Genes To Work for Positive Regulation under Low-Light Conditions in *Synechocystis* sp. Strain PCC 6803. Journal of Bacteriology 191, 1581–1586.

Suzuki I, Kanesaki Y, Mikami K, Kanehisa M, Murata N. 2001. Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. Molecular Microbiology 40, 235–244.

Takahashi T, Nakai N, Muramatsu M, Hihara Y. 2010. Role of Multiple HLR1 Sequences in the Regulation of the Dual Promoters of the *psaAB* Genes in *Synechocystis* sp. PCC 6803. 2010. Molecular Microbiology. 192, 4031–4036.

Takai N. 2006. A KaiC-associating SasA–RpaA two-component regulatory system as a major circadian timing mediator in cyanobacteria. 2006. Proceedings of the National Academy of Sciences of the United States of America 103, 2109-12114.

Tarassova OS, Chamot D. Owttrim GW. 2014. Conditional, temperature-induced proteolytic regulation of cyanobacterial RNA helicase expression. Journal of Bacteriology 193, 1560– 1568.

Umena Y, Kawakami K, Shen JR, Kamiya N. 2011. Crystal structure of oxygen evolving photosystem II at a resolution of 1.9 A. Nature. 473, 55-60.

van Beilen WA, Hellingwerf KJ. 2016. All Three Endogenous Quinone Species of Escherichia coli Are Involved in Controlling the Activity of the Aerobic/Anaerobic Response Regulator ArcA. Frontiers in Microbiology 7, 1402–11.

Xia Y and Xun L. 2017. Revised Mechanism and Improved Efficiency of the QuikChange Site-Directed Mutagenesis Method. Methods in Molecular Biology 1498, 367-374.

Zhang P Aro EM. 2012. Operon *flv4-flv2* Provides Cyanobacterial Photosystem II with Flexibility of Electron Transfer. Plant Cell 24, 1952–1971.

Zito I. 1999. The $Q_O$ site of cytochrome $b_6f$ complexes controls the activation of the LHCII kinase. EMMO. 18, 2961-2969.
.

# Chapter 5: Summary and Conclusion

Cyanobacteria are crucial primary producers worldwide, with over a third of amino acid turnover in marine environments facilitated by *Prochlorococcus* sp. and *Synechococcus* sp. alone (Zubkov et al., 2002). However, cyanobacteria can also form blooms having the potential to form a diverse range of toxic secondary metabolites, leading to a negative reputation and reduction of water quality. Understanding of cyanobacterial gene expression in response to changing environmental conditions is thus relevant both on an ecological scale, as well as a local one. Since chloroplasts evolved by endosymbiosis of a cyanobacterial progenitor (Raven and Allen 2003), the core photosynthetic apparatus, including the photosystems are highly conserved with higher plants however the light harvesting apparatuses differ significantly (Nelson and Ben-Shem, 2004). While generally cyanobacteria are obligate photoautotrophs, the glucose tolerant strain of *Synechocystis* can also be grown photomixotrophically, allowing for the study of photosynthetic mutants not viable in other organisms (Kaneko et al., 1996). Together with well-developed molecular tools and reasonable culture conditions, *Synechocystis* is a useful model system for the study of photosynthetic gene regulation.

The ability to sense and respond to changes in growth environment is essential for survival of photoautotrophic organisms. The crucial nature of this ability is evidenced by the extended genetic capacity of cyanobacteria to detect and respond physiologically to changes in their environment. *Synechocystis* encodes for 47 histidine kinase sensors (HKs) which coordinate cellular response to altered environmental conditions via 42 response regulators (RRs) (Murata and Suzuki, 2006). In addition to other stresses, and similar to other bacteria, *Synechocystis* utilizes HKs and RRs to respond to temperature fluctuation. Elevated temperature induces heat shock proteins (HSP) such as the protein chaperones *groEL*/*groES* (Suzuki et al., 2005) and induction of *groEL* at the mRNA level can be detected minutes after changes in temperature, allowing for rapid adaptation. Recently, some of the classic cold-induced genes previously thought to be temperature responsive, such as the acyl lipid desaturases (*desA, desB, desD*) responsible for maintaining membrane fluidity, are now known to be dependent on light for expression (Chen et al., 2018). Of the 36 tested genes identified as cold responsive, 19 were light dependent, suggesting coordination of light availability with temperature response is more widespread than previously expected.

Sensing and responding to light is a fundamental requirement for survival of obligate photoautotrophs such as cyanobacteria. A diverse range of light sensing and response mechanisms have therefore evolved in these organisms. Chromatic acclimation, the process by which cyanobacteria adjust the pigment composition of the light harvesting phycobilisomes, is mediated directly by light responsive photoreceptor proteins (Gutu and Kehoe, 2011). The complement of phytochrome-family photoreceptors is expanded in *Synechocystis* (Wiltbank and Kehoe., 2019). Unlike higher plants, cyanobacterial photoreceptors often contain multiple photosensory domains, allowing integration of multiple light signals through a single sensor. A second major mechanism by which the light environment is perceived involves detection of light-driven alterations in the redox potential of the electron transport chain (ETC). Redox regulation differs from processes directly sensitive to light quality, which operate independently of the ETC (Wiltbank and Kehoe, 2019). Various redox regulated genes have been described in cyanobacteria, including *psbA*, which encodes the D1 protein essential for forming the PSII reaction center (Alfonso et al., 2000). Redox regulation has largely been described to be contingent on the redox poise of the PQ pool in *Synechocystis*, primarily due to widespread utilization of DCMU and DBMIB (Mullineux and Allen., 1986; Komenda and Barber., 1996; Kujat and Owttrim 2000; Ritter et al., 2019). However, DCMU and DBMIB fail to differentially affect the redox poise PQ and of the upstream electron acceptor, $Q_B$. The focus of this thesis is to elucidate the mechanism by which *Synechocystis* detects a diverse range of abiotic stresses through their common effect on the redox potential of the ETC which regulates expression of the DEAD-box RNA helicase *crhR*.

Although *crhR* is primarily utilized as a reporter gene here, it is important to remember that through RNA helicase activity, CrhR can elicit changes in gene expression whose products facilitate cellular adaptation to abiotic stress. Changes in gene expression occur through helicase activity able to both unwind and anneal short RNA substrates (Chamot et al., 2005). The physiological importance of CrhR is indicated by deletion strains exhibiting a severe cold phenotype involving reduced viability and alterations in cellular morphology, photosynthetic electron flow and photosynthetic pigment composition (Rosana et al., 2012). Potential targets of the RNA helicase activity responsible for defects in photosynthesis include the flavodiiron proteins *flv3/4* and the ncRNA PmgR1, both of

which are misregulated in the absence of CrhR (Georg et al., 2019). Thus, CrhR has been previously demonstrated to act as a crucial regulator of photosynthesis in response to low temperature stress. By characterizing the complex mechanism of *crhR* regulation which requires perception of abiotic stress effects through a redox mediated convergent sensing mechanism, the essential role in maintenance of photosynthesis under abiotic stress was further elucidated. Further investigation of light-related regulatory mechanisms acting at multiple levels of gene expression and signal transduction from $Q_B$ via a two-component signal transduction mechanism involving Hik8-RpaA-RpaB further strengthen the connection between perception of environmental growth conditions and gene regulation.

## 5.1 – CrhR responds to diverse abiotic stresses through common effects on the ETC redox poise

*crhR* had previously been demonstrated to be responsive to the redox poise of the ETC through divergent effects of the inhibitors DCMU and DBMIB. At normal growth temperature, 30ºC, DCMU causes oxidation of $Q_B$/PQ and decreased expression, while DBMIB favors reduction and enhanced CrhR accumulation (Kujat and Owttrim, 2000). The crucial role of the ETC redox poise was further supported by a range of experiments. As aspects of the respiratory and photosynthetic ETCs are shared in *Synechocystis*, rescue of dark induced repression of CrhR using glucose, which in cyanobacteria directly contributes respiratory-derived electrons to PQ through NADPH reductase suggests involvement of the ETC (Hirano et al., 1980). The crucial role of the ETC for glucose rescue was confirmed by the failure of 3-OMG a glucose analog which can be transported but not actively metabolized by *Synechocystis* failing to rescue expression of *crhR* (Flores and Schmetterer, 1986; Kujat and Owttrim, 2000). Finally, alteration of light quality to stimulate PSII and PSI demonstrated that *crhR* expression required reduction of the area around PQ but was not affected by the redox status downstream of this location (Kujat and Owttrim, 2000).

In Chapter 2, the interaction between diverse abiotic stresses, the redox poise of the ETC and gene regulation by utilizing CrhR as a reporter were investigated. A major observation involved demonstration that CrhR expression is regulated by the redox poise of the ETC and is not directly responsive to any single abiotic stress. Instead, a range of

techniques demonstrated that diverse stresses, all of which have a common effect on reduction of the ETC, facilitated induction of CrhR expression (Ritter et al., 2019). Salt (NaCl), osmotic (sorbitol), and heavy metal ($ZnCl_2$, $CoCl_2$) all cause linear induction of CrhR with similar kinetics observed in response to cold stress. Reactive oxygen species (ROS) are natural byproduct of photosynthesis commonly utilized to enact changes in gene expression (Schmitt et al., 2014). As a result, it was crucial to differentiate between ETC redox regulation and potential regulation by the concomitant formation of ROS. Oxidative stressors $H_2O_2$ and rose bengal, which are not expected to influence the redox poise of the ETC, caused transient changes in CrhR expression despite causing significantly elevated levels of ROS. Thus, regulation of CrhR is mediated by multiple stressors through alteration of the redox poise of the ETC, an effect that is not contingent on ROS signaling. The resulting hypothesis, convergent stress sensing, is novel as while redox regulation controls expression of proteins which facilitate adaptation to specific abiotic stress, it has not been demonstrated that diverse abiotic stresses can be perceived through common effects on the redox poise of the ETC.

Instead, using the aforementioned inhibitors DCMU and DBMIB, induction of CrhR via diverse abiotic stresses was shown to universally require the ability for $Q_B$/PQ to be reduced. PSI does not play a role in this mechanism of redox regulation, as the PSI electron acceptor methyl viologen (MV) had little effect on *crhR* expression. Importantly, the predicted enhanced reduction or oxidation effect on the redox poise of the ETC in the vicinity of PQ was detected for all stresses previously demonstrated to induce CrhR using Pulse Amplitude Modulation (PAM) fluorometry. Similarly, the effect of all utilized ETC inhibitors were also confirmed. PAM analysis indicated that abiotic stresses shown here to induce CrhR expression were also associated with decreased photochemical quenching (qP), which indicated that PSII reaction centers are relatively more closed i.e. reduced (Campbell et al., 1998). A decrease in qP was not observed for oxidative stresses which failed to affect CrhR expression, confirming the redox poise of the ETC itself and not ROS generated as a byproduct of photosynthesis is responsible for induction of CrhR. Importantly, stress alteration of the ETC redox poise occurred within minutes of stress application and persisted for the remainder of the experiment, up to three hours, similar timescales as observed for CrhR induction. The proposed convergent sensing mechanism is

also different from other redox-regulated systems since increased reduction of PSII was not associated with induction of nonphotochemical quenching (NPQ), suggesting that the mechanisms which control state transition and induction of orange carotenoid protein, both of which are required for NPQ in cyanobacteria, are separate from those which regulate CrhR (Misumi et al., 2016). *psbA,* which encodes for the D1 subunit of PSII, has been demonstrated to be regulated in a similar redox dependent fashion (Alfonso et al., 2000). An additional layer of *psbA* regulation occurs post-transcriptionally, with the antisense RNA PsbA2R protecting the *psbA2* mRNA from degradation by RNAse E in the light, but not dark (Sakurai et al., 2012). How association and release of the asRNA from *psbA2* occurs is not known, but would presumably rely upon the activity of a RNA helicase such as *crhR.*

By using the redox poise of the ETC to perceive diverse abiotic stress, CrhR allows for coordinated changes in photosynthetic gene expression, maintaining electron transport under environmental conditions that result in overreduction of the ETC and thus helping to prevent photoinhibition. Similarly, CrhR has previously been demonstrated to contribute to regulation of various photosynthetic proteins, leading to defects in electron transport activity and decreased viability under temperature stress (Rosana et al., 2012; Georg et al., 2019). Further insights into the physiological roles performed by CrhR would involve determination of photoinhibition levels and rates of D1 turnover that should be investigated in strains lacking CrhR.

A major conclusion of this manuscript involved description of redox regulation as a convergent sensing mechanism by which diverse abiotic stresses are perceived. Convergent sensing facilitates response to diverse abiotic stresses through a shared indirect effect on the redox poise of the ETC. This is in contrast to the more canonical two-component convergent signaling systems present in bacteria, where independent signaling pathways unique for each stress operate in parallel, leading to redundant or conflicting effects at the level of gene expression. Unlike other bacterial systems, individual two-component signaling systems do not regulate the entire cold, salt or osmotic stress responsive regulons in *Synechocystis.* Thus, convergent sensing systems may account for changes in expression of these previously "orphan" genes which lack any established regulators (Suzuki et al., 2001; Mikami et al., 2002; Marin et al., 2003). From an evolutionary aspect, related

systems have been demonstrated in bacteria and chloroplasts suggesting regulation by convergent sensing may be more widespread than previously thought (Zito et al., 1999; van Bielen and Hellingwerf, 2016).

## 5.2 – Regulation of CrhR is contingent on three independent light signals

In Chapter 3 the previously demonstrated redox regulation of CrhR is expanded upon by investigating the role of light quality in gene regulation at multiple levels of expression. The relative contribution of light, redox and ROS were evaluated using all combinations of light and oxygen stress in an interlinked experimental setup as previously described by Barth *et al*., (2014). This analysis confirmed that expression of CrhR responds to the redox poise of the ETC but is also contingent upon the presence of a permissive light signal for expression. This crucial observation was further supported by the ability of high light to induce CrhR expression, an effect that was contingent on the redox status of $Q_B$/PQ, as previously described (Kujat and Owttrim, 2000; Ritter et al., 2019). Thus, a light signal is required to induce electron flow through the ETC that facilitates induction of CrhR in a redox dependent mechanism.

Investigation into the crucial role of light raised questions as to how signals arising from light directly and light-driven changes in the redox poise of the ETC act together to regulate CrhR expression. As described above in Section 5.1, reduction of $Q_B$/PQ is essential to permit abiotic stress mediated induction of CrhR at the protein level (Ritter et al., 2019). It has previously been demonstrated that light-driven changes in the redox poise of PQ is the initial light signal required for expression at the level of transcript accumulation (Kujat and Owttrim, 2000). Here we confirmed these results, as glucose was able to increase expression of *crhR* transcript accumulation in the dark, presumably through contribution of respiratory electrons to the ETC at PQ in *Synechocystis* (Hirano et al., 1980). As a result, light is not a direct requirement for expression of *crhR* at the transcript level, it is instead indirectly required for reduction of the ETC which is oxidized in the dark (Schuurmans et al., 2014). This mechanism is supported by induction of *crhR* transcript to a maximum level in the presence of all tested wavelengths of light, which unexpectedly does not occur at the protein level.

At the protein level, a second signal dependent on specific wavelengths of light is required for translation of CrhR. Here, CrhR protein accumulation only occurred in the presence of red and white, but not blue light. This suggests that, unlike at the transcriptional level, translation is not responsive to the redox poise of the ETC. Instead, as CrhR contains no known photosensitive domains, this suggests translation initiation requires a permissive signal via a protein capable of sensing specific wavelengths of light directly. In cyanobacteria, phytochrome superfamily photoreceptors are expanded and commonly utilize multiple light sensory domains, indicative of the crucial role light plays in metabolism (Wiltbank and Kehoe, 2019). Similar examples of light quality regulation and the post-transcriptional level are rare, with regulation of protochlorophyllide reductase (PORA) in *Arabidopsis* providing the most analogous example (Paik et al., 2012). In this case, the RNA-binding protein Pnt1 recruits the functionally redundant phytochromes PhyA/B to the PORA mRNA via interactions at the 5'UTR. Upon exposure to light, photoconversion of PhyA/B causes release of the complex, allowing translation to proceed. Since CrhR is an RNA binding protein, it may perform an analogous function similar to Pnt1 in *Synechocystis,* as interactions between CrhR and the *por* mRNA homologous to *Arabidopsis* PORA have been detected (Wolfgang Hess, personal communication). Further investigation into interactions between CrhR and phytochrome family proteins using techniques to detect protein-protein interactions, such as BioID, are ongoing in the Owttrim lab (Whitman, unpublished).

Differential expression of *crhR* in blue light at the transcript and protein levels suggests that CrhR functionality is required in red and white, but not blue light. This requirement appears to be a direct reflection of the normal habitat combined with the mechanism by which *crhR* expression is regulated. Cyanobacteria primarily use phycobilisomes for light harvesting, which have negligible absorption of wavelengths below 495 nm (Grossman et al., 1993). As a result, it has been recently shown in *Synechocystis* that while blue light (PSI) is absorbed to a similar extent to red light (PSII), blue light is utilized less effectively for oxygenic photosynthesis and growth (Luimstra et al., 2018). Thus, red light will stimulate reduction of $Q_B$/PQ more efficiently than blue light, a requirement for *crhR* expression The data therefore suggest that CrhR RNA helicase activity is required only in conditions when the ETC has the potential to become

over-reduced. This is further supported by induction of CrhR in response to abiotic stress in response to increasd reduction of PSII (Ritter et al., 2019). CrhR may function to induce systems evolved to mitigate the effects of excess light-derived electrons, a process termed photoinhibition. As such, deletion of CrhR caused changes in expression of the flavodiiron proteins (*flv2/3/4)* which aid in photoprotection of PSII and PmgR1 a non-coding RNA crucial for regulation of glucose metabolism, again confirming a crucial role for CrhR RNA-helicase activity in the maintenance of photosynthesis (Zhang et al., 2012; de Porcellinis et al., 2016; Georg et al., 2019).

Finally, the response of *crhR* expression to the absence of light yielded unique insights. Despite being placed at temperatures which would favor either expression or proteolysis of CrhR, neither of these processes where induced in the dark, an explanation that cannot be explained by redox regulation. As proteolysis of CrhR had been previously demonstrated to proceed independently of the redox status of $Q_B$/PQ, this further confirms that induction and repression of CrhR are controlled by separate regulatory mechanisms (Ritter et al,, 2019).

A crucial insight into the regulatory mechanism was revealed by the observation that CrhR expression in the presence of antibiotics demonstrated that translation of CrhR was pre-initiated at 30$^{o}$C. This suggested the existence of an additional layer of regulation at the level of translation elongation. A related system involves the light responsive synthesis of the crucial PSII protein D1 in *Synechocystis* (Tyystjärvi et al., 2001). Similar to D1 synthesis, where rapid replacement is essential for prevention of photoinhibition, pre-initiation of *crhR* would allow for rapid production of CrhR protein when under permissive conditions.

The data presented here can be interpreted to indicate that a minimum of three light signals are involved in CrhR regulation. The initial and most crucial signal is associated with induction involving the light driven changes in the redox poise of the ETC where reduction of PQ enhances CrhR accumulation, in agreement with previous data (Kujat and Owttrim, 2000; Ritter et al., 2019). Redox regulation of *crhR* is imparted at the transcriptional level, a signal that does not affect proteolysis, suggesting that divergent signaling mechanisms are required for both processes. The second and perhaps most unexpected signal is the requirement for specific wavelengths of light to allow for

translation of CrhR. Red light permitted translation of CrhR in response to cold shock both at a similar rate and to a similar maximum level as observed in white light. However, CrhR accumulation was impeded in the presence of blue light despite incubation at a temperature permissive to expression. This suggested involvement of a phytochrome-like protein which responds to a specific wavelength of light. The final signal is a light signal which is wavelength- and redox-independent and is associated with the proteolytic repression of CrhR induction in the absence of stress. Finally, dark conditions superseded the temperature signal for the transcription, translation and proteolytic degradation of CrhR. While we anticipate that the dark effect is directly associated with the distinct light signals required for each of these processes, the nature of the dark regulation mechanism requires further investigation. Dark regulation occurs independently of redox regulation via the ETC at the transcriptional level and a phytochrome family mediated mechanism at the translational level, as both presence of ETC inhibitors and varying wavelengths of light do not affect proteolysis. This observation is unique since while proteolysis in response to varying light quality has been described (Henriques et al., 2009; Lemeille et al., 2010), a requirement for light to permit proteolysis in response to changes in temperature has not previously been demonstrated. The requirement for an additional layer of regulation which prevents proteolysis in the dark implies CrhR may be required for both light-dark and dark-light transitions and thus suggest a role for CrhR in regulation of the circadian clock in *Synechocystis* (Köbler et al., 2018). Expression of *crhR* is also responsive to diverse abiotic stresses through their indirect effect on the redox poise of the ETC (Ritter et al., 2019). Lack of functional CrhR leads to misregulation of genes involved in both photosynthesis and energy metabolism, which manifest as reduced electron transport and increased photoinhibition (Rosana et al., 2012; Georg et al., 2019). Thus, CrhR appears to be a key regulator of photosynthesis through coordination of input from both abiotic stress effects and altered light availability. However, the interacting genetic components which facilitate these regulatory mechanisms remain unidentified.

## 5.3 – Expression of CrhR is responsive to $Q_B$ and not PQ

In Chapter 3, the specific location of the ETC responsible for perception of abiotic stress through alteration in redox poise, as well as the signal transduction mechanism which links

the ETC with CrhR induction was investigated. Despite the knowledge that multiple redox responsive signaling systems exist in *Synechocystis,* how changes in the redox poise of the ETC is coupled with regulation of CrhR expression and other redox regulated genes is still unclear. DCMU and DBMIB have been proven to act in a site-specific fashion leading to differential oxidation of the ETC generally interpreted to indicate the redox poise of PQ as crucial for gene regulation (Calzadilla et al., 2019; Khorobrykh et al., 2019; Ritter et al., 2019). However, the area between $Q_A$ (the site of DCMU binding) and $Q_O$ in cyt-*b6f* which consist of $Q_B$ within PSII and the freely lipid-diffusible PQ-pool within the thylakoid membrane, will also be universally oxidized by DCMU and reduced by DBMIB. As a result, although studies utilizing these inhibitors commonly interpret that PQ is the primary site of regulation, this potentially is an oversimplification with both $Q_B$ and PQ offering the potential to act as a site of redox sensing. In this section, evidence was presented indicating that $Q_B$ was the site of physiological change within the ETC responsible for providing the redox signal required for CrhR induction.

Only a few previous studies have interpreted that $Q_B$ is the ETC component whose redox poise is the sensor that activates a two-component signal transduction system to regulate downstream gene expression. In 1995, Komenda and Barber presented genetic data that indirectly suggested that $Q_B$ and not PQ was responsible for regulation of D1 turnover. This was supported by inhibition of the expected induction of D1 turnover in response to high light as a result of an increased rate of photooxidation at $Q_B$ caused by the prevention of electron flow out of $Q_B$ as a result of mutation of *psbH* (Komenda and Barber, 1995). In contrast, D1 turnover occurred normally when *psbO*, which also acts within PSII but does not accumulate photodamage at $Q_B$, was mutated. Thus, PSII mutants that allow reduction of $Q_B$ still demonstrated redox-dependent gene regulation. Unfortunately, utilization of these mutants to investigate the role of $Q_B$ in the redox regulation of CrhR in response to cold shock failed to repeat the pattern observed by Komenda and Barber for D1 regulation in response to high light (1995). These results are completely unexpected and most likely originate from a reduced level of photooxidative damage associated with cold stress compared to high light stress (Allakhverdiev and Murata, 2004). Thus, these experiments should be repeated under high light conditions to ensure that D1 photooxidative damage is enhanced especially in the *psbH* mutant. Under

these conditions it would be anticipated that *crhR* expression would be inhibited by *psbH* and not by *psbO* mutation.

However, utilization of quinones that function as electron sinks from different locations within the ETC clearly supported $Q_B$ regulation. It had previously been demonstrated that DCBQ and PPBQ but not DMBQ oxidize $Q_B$ (Fu et al., 2017). When coupled with ferricyanide to maintain the quinones in an oxidized state (Shevela and Messinger, 2012), DCBQ and PPBQ but not DMBQ were capable of preventing CrhR induction in response to cold shock. These results provided compelling evidence that the redox poise of $Q_B$ and not PQ regulates *crhR* expression.

Application of oxidized quinones has been shown to delay rhythmic oscillation in the phosphorylation state of the key circadian clock protein KaiC, confirming that redox changes facilitated by an expanded quinone pool can elicit changes in downstream gene regulation in a similar fashion to that observed for CrhR (Kim et al., 2012). The conclusion presented here indicating $Q_B$ as the redox sensor may also be the more logical mechanism from an evolutionary perspective since it is difficult to formulate a mechanism by which the average redox status of a freely lipid diffusible quinone such as PQ can be measured by a membrane-associated redox-responsive HK. In addition, previous data interpreted to involve regulation by the redox status of PQ, including state transitions, should be reevaluated for potential involvement of $Q_B$. In higher plants state transition, is responsive to the $Q_O$ site of cyt $b_6f$ (Zito et al., 1999). As state transition has been demonstrated to proceed independently of $Q_O$ in cyanobacteria, the $Q_B$ redox status may perform an extended role in gene regulation in cyanobacteria compared to higher plants (Calzadilla et al., 2019). In summary, utilization of quinones that alter the redox poise of electron carriers at specific positions within the ETC allowed us to identify $Q_B$ as the sensor whose overreduction elicits *crhR* expression. Reevaluation of redox regulated gene expression attributed to the redox poise of PQ should be conducted.

## 5.4 – Signal transduction downstream of $Q_B$/PQ redox poise

Irrespective of $Q_B$ or PQ as the ETC redox poise indicator, the nature of the two-component signaling system downstream of the ETC has not been identified. Unlike in

higher plants, Ser/Thr kinases as well as Tyr kinases do not facilitate signal transduction downstream of the ETC in cyanobacteria (Calzadilla et al., 2019). As a result, HKs and their corresponding RRs are likely candidates for linking the ETC with changes in expression of redox responsive genes such as *crhR.* Here, a series of HK and RR mutants known to be responsive to changes in ETC redox poise were systematically evaluated for altered *crhR* expression.

Despite *hik30* and *rre33* being responsive to DCMU and contributing to regulation of photosystem stoichiometry (Li and Sherman, 2000), both components of this two-component system did not alter CrhR in response to diverse abiotic stress. This result suggested that state transition and CrhR are both responsive to the redox poise of $Q_B$/PQ but proceed through independent two-component pathways. Similarly, other HK – RR systems known to be associated redox responses were also shown to have no effect on CrhR expression. This included the *hik2*/*rre1*/*rre33* system, whose association with PQ redox sensing had previously been postulated (Paithanoorangsarid et al, 2004). Hik2, although originally described as salt responsive, had been proposed to respond to the redox poise of PQ as it is highly homologous to Chloroplast Sensory Kinase (CSK) that conveys redox signals in higher plant chloroplasts (Paithanoorangsarid et al., 2004). Rre33 has also been proposed to act downstream of the essential gene Hik2 in conjunction with Rre1. Both RRs have been demonstrated to be directly phosphorylated by Hik2 with Rre1 outcompeting Rre33 (Ibrahim et al., 2016). As both *hik2* and *rre1* are essential, a *rre1* over-expression construct was utilized to investigate the effect of this signaling pathway on regulation of CrhR Overexpression of Rre1 was confirmed at the transcript level using RT-PCR and physiologically as overexpression lead to a significant increase in thermotolerance (Kaczmaryzyk et al., 2014). The rate of CrhR induction in response to temperature change was not altered by Rre1 overexpression. While this appears to suggest CrhR induction proceeds independently of Rre1, confirmation of overexpression at the transcript, protein or physiological levels is also required. However, deletion of Hik34, which activates Rre1 in response to heat shock (Vidal et al., 2009), also had no effect on CrhR expression, supporting a lack of involvement of Rre1.

Similar to Hik2, Hik33 has also been postulated to be responsive to the changes in the redox poise of PQ caused by both cold and osmotic stress (Mikami et al., 2002;

Shimura et al., 2012; Maksimov et al., 2017). Again, CrhR induction in response to changes in temperature proceeded normally in the absence of Hik33. This data actually confirms previous investigation of the regulons controlled by Hik33 at the transcriptomic and proteomic levels in response to both temperature and salt stress which did not identify *crhR* as a target (Suzuki et al., 2001; Ge et al., 2017).

The response regulator RpaA has been shown to respond to both the aforementioned Hik33 as well as Hik8 (Kuwahara et al., 2015). Hik8 is a SasA ortholog proposed to be the primary output of the KaiABC clock responsible for generation of circadian gene expression in *Synechocystis* (Iijima et al., 2015; Köbler et al., 2018). Genetic manipulation of Hik8 levels resulted in altered glucose metabolism and decreased viability when grown under light – dark cycles (Singh and Sherman, 2005; Tabei et al., 2012). Despite a lack of effect of *hik33* deletion on CrhR expression, deletion of *rpaA* caused a reduction, but not complete abolition of, the level of CrhR expression in response to both temperature and salt stress. These results suggested that crhR expression is regulated by the Hik8-RpaA two-component signal transduction pathway and indicated involvement of Hik8 through light – dark transitions and the KaiABC clock complex. This association is not unexpected, as CrhR regulation has been demonstrated to involve a complex network of interacting light signals, suggesting influence of circadian rhythm. However, there is the possibility alteration of CrhR expression in the Δ*rpaA* strain is a secondary effect as overexpression of RpaA has been demonstrated to alter photosynthetic electron transport (Arisaka et al., 2018).

RpaA is associated with gene regulation in response to high light stress in *Synechocystis* through the conserved promoter regulatory sequence HLR1 (Markson et al., 2013). Recently, a promoter element bearing striking resemblance to the consensus HLR1 site was identified upstream of *crhR* (Riedeger et al., 2018). To further investigate whether RpaA directly controls CrhR expression, the predicted HLR1 site was replaced with a random sequence of identical length. HLR1 is also proposed to interact with RpaB, an essential gene which has been described as playing a similar role as RpaA in light acclimation and circadian-clock related processes (Piechura et al., 2017). RpaA and RpaB have also been shown to interact to coordinate gene expression, with overexpression of a phosphorylation defective mutant of RpaB rescuing a light – dark cycle sensitive

phenotype caused by deletion of *rpaA* (Espinosa et al., 2015). Ablation of the potential HLR1 site predicted to interact with RpaA unfortunately did not prevent CrhR induction in response to temperature. This suggests two possible explanations for the inhibition of CrhR expression in the Δ*rpaA* stain: 1) Deletion of RpaA causes changes in the redox poise of the ETC, which indirectly effects CrhR expression and thus CrhR induction relies on a yet to be identified signaling system or 2) Hik8 is the actual $Q_B$/PQ sensor that requires interaction between the two RRs. In the absence of RpaA, RpaB is not completely activated and thus cannot regulate *crhR* transcription correctly, leading to the observed partial inhibition of expression. Unfortunately, we cannot analyze the role of RpaB directly as it is an essential gene. Further insight into the role of these essential genes could be achieved through CRIPSRi facilitated inducible knockdowns (Yao et al., 2015; Kaczmarzyk et al., 2017).

Redox regulation of CrhR is crucial for the adaptation of photosynthesis in response to abiotic stress (Rosana et al, 2012; Ritter et al, 2019). Previously suggested to be responsive to changes in the $PQH_2$ pool (Kujat and Owttrim, 2000), we propose that $Q_B$ is instead responsible for induction of *crhR*. Genetic analysis indicates that the $Q_B$ redox poise is transmitted to the *crhR* promoter via a two-component signal transduction chain involving RpaA and presumably, Hik8. Since complete abolition of CrhR induction by various stresses was not observed in the RpaA mutant, this suggests that the observed effects are caused indirectly via alteration of photosynthetic electron flow or via potential compensation of RpaB in the absence of RpaA. Unfortunately, since both Hik8 and RpaB are essential genes in *Synechocystis* (Takahashi et al., 2010; Kuwahara et al., 2015), we cannot confirm this hypothesis directly using genetic knockouts. In conjunction, *in vivo* promoter pulldowns would provide information on the potential interactions of RpaA/RpaB with the *crhR* promoter required to elicit *crhR* expression. Investigation into the signaling pathways required for expression of CrhR will reveal further insight into how redox, light and circadian rhythm responsive gene mechanisms work together to facilitate adaptation to changing environmental conditions.

## 5.5 References

Alfonso A, Perewoska I, Kirilovsky D. 2000. Redox Control of *psbA* Gene Expression in the Cyanobacterium *Synechocystis* PCC 6803. Involvement of the Cytochrome $b_6f$ Complex. Plant Physiology 122, 505-515.

Allakhverdiev S, Murata N. 2004. Environmental stress inhibits the synthesis de novo of proteins involved in the photodamage–repair cycle of Photosystem II in *Synechocystis* sp. PCC 6803. Biochimica Et Biophysica Acta - Bioenergetics 1657, 23–32.

Barth J, Bergner SV, Jaeger D, Niehues A, Schulze S, Scholz M, Fufezan C. 2014. The interplay of light and oxygen in the reactive oxygen stress response of *Chlamydomonas reinhardtii* dissected by quantitative mass spectrometry. Molecular and Cellular Proteomics 13, 969–989.

Calzadilla PI, Zhan J, Sétif P, Lemaire C, Solymosi D, Battchikova N, Wang Q, Kirilovsky D. 2019. The cytochrome $b_6f$ complex is not involved in cyanobacterial state transitions. Plant Cell 31, 911–931.

Campbell D, Hurry V, Clarke AK, Gustafsson P, Oquist G. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiology and Molecular Biology Reviews 62, 667–683.

Chamot D, Colvin KR, Kujat-Choy SL, Owttrim GW. 2005. RNA structural rearrangement via unwinding and annealing by the cyanobacterial RNA helicase, CrhR. Journal of Biological Chemistry 280, 2036–2044.

Chen W, Fang L, Huang X, Ge H, Wang J, Wang X, Zhang Y, Sui N, Xu W, and Wang Y. 2018. Systematic identification of light-regulated cold-responsive proteome in a model cyanobacterium. Journal of Proteomics 179, 100–109.

de Porcellinis AJ, Klähn S, Rosgaard L, Kirsch R, Gutekunst K, Georg, J, Hess W. 2016. The Non-Coding RNA Ncr0700/PmgR1 is Required for Photomixotrophic Growth and the Regulation of Glycogen Accumulation in the Cyanobacterium *Synechocystis* sp. PCC 6803. Plant and Cell Physiology 57, 2091–2103.

Espinosa, J, Boyd JS, Cantos R, Salinas P, Golden SS, Contreras A. 2015. Crosstalk and regulatory interactions between the essential response regulator RpaB and cyanobacterial circadian clock output. Proceedings of the National Academy of Sciences of the United States of America 112, 7.

Fu HY, Picot D, Choquet Y, Longatte G, Sayegh A, Delacotte J, Wollman FA. 2017. Redesigning the $Q_A$ binding site of Photosystem II allows reduction of exogenous quinones. Nature communications 8, 15274.

Ge H. 2017 Translating Divergent Environmental Stresses into a Common Proteome Response through the Histidine Kinase 33 (Hik33) in a Model Cyanobacterium. 2017. Journal of Proteomics 16, 1258–1274.

Georg J, Rosana ARR, Chamot D, Migur A, Hess WR, Owttrim GW. 2019. Inactivation of the RNA helicase CrhR impacts a specific subset of the transcriptome in the cyanobacterium *Synechocystis* sp. PCC 6803. RNA Biology 16, 1205–1214.

Gutu A, Kehoe DM. Emerging Perspectives on the Mechanisms, Regulation, and Distribution of Light Color Acclimation in Cyanobacteria. 2011. Nature Reviews 5, 1–13.

Grossman AR, Schaefer MR, Chiang GG, Collier JL. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiology Reviews. 57, 725– 749.

Henriques R, Jang I, Chua N. Regulated proteolysis in light-related signaling pathways. 2009. Current Opinions in Plant Biology 12, 49–56.

Hirano M, Satoh K, Katoh, S. 1980. Plastoquinone as a common link between photosynthesis and respiration in blue-green alga. Photosynthesis Research 1, 149–162.

Ibrahim IM, Puthiyaveetil S, Allen JF. 2016. A two-component regulatory system in transcriptional control of photosystem stoichiometry: Redox-dependent and sodium ion-dependent phosphoryl transfer from cyanobacterial histidine kinase Hik2 to response regulators Rre1 and RppA. Frontiers in Plant Science 12, 196–212.

Iijima H, Shirai T, Okamoto M, Kondo A, Hirai MY, Osanai T. 2015. Changes in primary metabolism under light and dark conditions in response to overproduction of a response regulator RpaA in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, Frontiers in Microbiology 6, 888.

Kaczmarzyk D, Anfelt J, Särnegrim A, Hudson EP. 2014. Overexpression of sigma factor SigB improves temperature and butanol tolerance of *Synechocystis* sp. PCC6803. Journal of Biotechnology, 182, 54–60.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Research 3,109–136.

Khorobrykh S, Tsurumaki T, Tanaka K, Tyystjärvi T, Tyystjärvi E. 2019. Measurement of the redox state of the plastoquinone pool in cyanobacteria. FEBS Letters 33, 1233–9.

Kim YI, Vinyard DJ, Ananyev GM, Dismukes GC, Golden SS. 2012. Oxidized quinones signal onset of darkness directly to the cyanobacterial circadian oscillator. Proceedings of the National Academy of Sciences of the United States of America USA 109, 17765–17769.

Köbler C, Schultz SJ, Kopp D, Voigt K, Wilde A. 2018. The role of the *Synechocystis* sp. PCC 6803 homolog of the circadian clock output regulator RpaA in day- night transitions. Molecular Microbiology 110, 847–861.

Komenda J, Barber J. 1995. Comparison of psbO and psbH deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the QB site and dependent on protein synthesis. Biochemistry, 34, 9625–9631.

Kujat SL, Owttrim GW. 2000. Redox-Regulated RNA Helicase Expression. Plant Physiology 124, 703-714.

Kuwahara A, Arisaka S, Takeya M, Iijima H, Hirai MY, Osanai T. 2015. Modification of photosynthetic electron transport and amino acid levels by overexpression of a circadian-related histidine kinase *hik8* in *Synechocystis* sp. PCC 6803. Frontiers in Microbiology 6, 637–9.

Li H, Sherman LA. 2000. A redox-responsive regulator of photosynthesis gene expression in the cyanobacterium *Synechocystis* sp. Strain PCC 6803. Journal of Bacteriology 182, 4268–4277.

Luimstra VM, Schuurmans JM, Verschoor AM, Hellingwerf KJ, Huisman J, Matthijs HCP. 2018. Blue light reduces photosynthetic efficiency of cyanobacteria through an imbalance between photosystems I and II. Photosynthesis Reseach. 138, 77–189.

Maksimov EG, Mironov KS, Trofimova MA, Nechaeva NL, Todorenko DA, Klementiev KE, Tsoraev GV, Tyutyaev EV, Zorina AA, Feduraev PV, Allakhverdiev SI, Paschenko VZ, Los DA. 2017. Membrane fluidity controls redox-regulated cold stress responses in cyanobacteria. Photosynthesis Research 133, 215–223.

Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N. 2003. Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis* sp. PCC 6803. Proceedings of the National Academy of Sciences of the United States of America USA 100, 9061–9066.

Markson JS, Piechura JR, Puszynska AM, O'Shea EK. 2013. Circadian Control of Global Gene Expression by the Cyanobacterial Master Regulator RpaA. Cell 155, 1396–1408.

Mikami K, Kanesaki Y, Suzuki I, Murata N. 2002. The histidine kinase Hik33 perceives osmotic stress and cold stress in *Synechocystis* sp PCC 6803. Molecular Microbiology 46, 905–915.

Misumi M, Katoh H, Tomo T, Sonoike K. 2016. Relationship Between Photochemical Quenching and Non-Photochemical Quenching in Six Species of Cyanobacteria Reveals Species Difference in Redox State and Species Commonality in Energy Dissipation. Plant and Cell Physiology 57, 1510–1517.

Mullineaux CW, Allen JF. 1986. The state 2 transition in the cyanobacterium *Synechococcus* 6301 can be driven by respiratory electron flow into the plastoquinone pool. FEBS Letters. 205, 155-160.

Murata N, Suzuki I. 2006. Exploitation of genomic sequences in a systematic analysis to access how cyanobacteria sense environmental stress. Journal of Experimental Botany 57, 235–247.

Nelson N, Ben-Shem A. 2004. The complex architecture of oxygenic photosynthesis. Nature Reviews 5, 971–982.

Paik I, Yang S, Choi, G. 2012. Phytochrome regulates translation of mRNA in the cytosol. Proceedings of the National Academy of Sciences of the United States of America USA 109, 335–1340.

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I, et al. 2004. Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. Journal of Biological Chemistry 279, 53078–53086.

Piechura JR, Amarnath K, O'Shea EK. 2017. Natural changes in light interact with circadian regulation at promoters to control gene expression in cyanobacteria, eLife. 6, e32032.

Raven JA, Allen JF. 2003. Genomics and chloroplast evolution: what did cyanobacteria do for plants? Genome Biology 4, 209.

Riediger M. 2019. Biocomputational Analyses and Experimental Validation Identify the Regulon Controlled by the Redox- Responsive Transcription Factor RpaB. 2019. iScience 15, 316–331.

Ritter SPA, Lewis AC, Vincent S., Lo LL, Cunha APA, Chamot D, Ensminger I, Espie GS. Owttrim GW. 2019. Evidence for convergent sensing of multiple abiotic stresses in cyanobacteria. Biochemica et Biophysica Acta. 1864, 129462.

Rosana AR, Chamot D, Owttrim GW. 2012a. Autoregulation of RNA helicase expression in response to temperature stress in *Synechocystis* sp. PCC 6803. PLoS ONE 7, e48683.

Shevela D, Messinger J 2012. Probing the turnover efficiency of photo- system II membrane fragments with different electron acceptors. Biochimica et Biophysica Acta 1817, 208–1212

Schuurmans RM, Schuurmans JM, Bekker M, Kromkamp JC, Matthijs HCP, Hellingwerf KJ. 2014. The Redox Potential of the Plastoquinone Pool of the Cyanobacterium *Synechocystis* Species Strain PCC 6803 Is under Strict Homeostatic Control. Plant Physiology 165, 463–475.

Shimura Y, Shiraiwa Y, Suzuki I. 2012. Characterization of the subdomains in the N-terminal region of histidine kinase Hik33 in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant and Cell Physiology 53, 1255–1266.

Singh AK, Sherman LA. 2005. Pleiotropic effect of a histidine kinase on carbohydrate metabolism in *Synechocystis* sp. strain PCC 6803 and its requirement for heterotrophic growth. Journal of Bacteriology 187, 2368–2376.

Suzuki I, Kanesaki Y, Mikami K, Kanehisa M, Murata N. 2001. Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. Molecular Microbiology 40, 235–244.

Suzuki I. 2005. The Histidine Kinase Hik34 Is Involved in Thermotolerance by Regulating the Expression of Heat Shock Genes in *Synechocystis*. 2005. Plant Physiology 138, 1409–1421.

Tabei I. 2012. Two Regulatory Networks Mediated by Light and Glucose Involved in Glycolytic Gene Expression in Cyanobacteria. 2012. Journal of Proteomics 53, 1720–1727.

Takahashi T, Nakai N, Muramatsu M, Hihara Y. 2010. Role of Multiple HLR1 Sequences in the Regulation of the Dual Promoters of the *psaAB* Genes in *Synechocystis* sp. PCC 6803. 2010. Molecular Microbiology. 192, 4031–4036.

Tyystjärvi T, Herranen M, Aro EM. 2001. Regulation of translation elongation in cyanobacteria: membrane targeting of the ribosome nascent-chain complexes controls the synthesis of D1 protein. Molecular Microbiology 240, 476–484.

van Beilen WA, Hellingwerf KJ. 2016. All Three Endogenous Quinone Species of Escherichia coli Are Involved in Controlling the Activity of the Aerobic/Anaerobic Response Regulator ArcA. Frontiers in Microbiology 7, 1402–11.

Vidal R, Lopez-Maury L, Guerrero MG, Florencio FJ. 2009. Characterization of an Alcohol Dehydrogenase from the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 That Responds to Environmental Stress Conditions via the Hik34-Rre1 Two-Component System. Journal of Bacteriology 191, 4383–4391.

Wiltbank LB, Kehoe DM. 2016. Two cyanobacterial photoreceptors regulate photosynthetic light harvesting by sensing teal, green, yellow and red light. mBio 7, e02130– 15.

Zhang P Aro EM. 2012. Operon *flv4-flv2* Provides Cyanobacterial Photosystem II with Flexibility of Electron Transfer. Plant Cell 24, 1952–1971.

Zito I. 1999. The $Q_O$ site of cytochrome $b_6f$ complexes controls the activation of the LHCII kinase. EMMO. 18, 2961-2969.

Zubkov MV, Fuchs BM, Tarran GA, Burkill PH, Amann R. 2003. High Rate of Uptake of Organic Nitrogen Compounds by Prochlorococcus Cyanobacteria as a Key to Their Dominance in Oligotrophic Oceanic Waters. Applied and Environmental Microbiology 69, 1299–1304.

# References

Alfonso A, Perewoska I, Kirilovsky D. 2000. Redox Control of *psbA* Gene Expression in the Cyanobacterium *Synechocystis* PCC 6803. Involvement of the Cytochrome $b_6f$ Complex. Plant Physiology 122, 505-515.

Allahverdiyeva Y, Mustila H, Ermakova M, Bersanini L, Richaud P, Ajlani G. 2013. Flavodiiron proteins Flv1 and Flv3 enable cyanobacterial growth and photosynthesis under fluctuating light. Journal of Bacteriology 110, 4111–4116.

Allakhverdiev S, Murata N. 2004. Environmental stress inhibits the synthesis de novo of proteins involved in the photodamage–repair cycle of Photosystem II in *Synechocystis* sp. PCC 6803. Biochimica Et Biophysica Acta - Bioenergetics 1657, 23–32.

Allen JF. 2017. The CoRR hypothesis for genes in organelles. Journal of theoretical biology 434, 50-57.

Anderson SL, McIntosh L. 1991. Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: A blue-light-requiring process. Journal of Bacteriology 173, 2761–2767.

Ashby MK, Houmard J. 2006. Cyanobacterial two-component proteins: structure, diversity, distribution, and evolution. Microbiology and Molecular Biology Reviews 70, 472–509.

Ashby MK, Mullineaux CW. 1999. Cyanobacterial *ycf27* gene products regulate energy transfer from phycobilisomes to photosystems I and II. FEMS Microbiology Letters 181, 253–260.

Baena-González E, Rolland F, Thevelein JM, and Sheen, J. 2007. A central integrator of transcription networks in plant stress and energy signalling. Nature 448, 938–942.

Baier A, Winkler W, Korte T, Lockau W, Karradt A. 2014. Degradation of phycobilisomes in *Synechocystis* sp. PCC6803: evidence for essential formation of an NblA1/NblA2 heterodimer and its codegradation by A Clp protease complex. Journal of Biological Chemistry 289, 11755–11766.

Balasubramanian R, Shen G, Bryant DA, Golbeck JH. 2006. Regulatory roles for IscA and SufA in iron homeostasis and redox stress responses in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Journal of Bacteriology 188, 3182–3191.

Ballut L, Marchadier B, Baguet A, Tomasetto C, Séraphin B, Le Hir H. 2005. The exon junction core complex is locked onto RNA by inhibition of eIF4AIII ATPase activity. Nature structural and molecular biology 12, 861.

Barth J, Bergner SV, Jaeger D, Niehues A, Schulze S, Scholz M, Fufezan C. 2014. The interplay of light and oxygen in the reactive oxygen stress response of *Chlamydomonas*

*reinhardtii* dissected by quantitative mass spectrometry. Molecular and Cellular Proteomics 13, 969–989.

Baymann F, Brugna M, Mühlenhoff U, Nitschke W. 2001. Daddy, where did PSI come from? Biochemica et Biophysica Acta 1507, 291–310.

Beier D, Gross R. 2006. Regulation of bacterial virulence by two-component systems. Current Opinion in Microbiology 9, 143–152.

Belatik A, Joly D, Hotchandani S, Carpentier R. 2013. Re-evaluation of the side effects of cytochrome $b_6f$ inhibitor dibromothymoquinone on photosystem II excitation and electron transfer. Photosynthesis Research 117, 489–496.

Berla BM, Saha R, Maranas CD, Pakrasi HB. 2015. Cyanobacterial alkanes modulate photosynthetic cyclic electron flow to assist growth under cold stress. Scientific Reports 5, 14894.

Bersanini L, Battchikova N, Jokel M, Rehman A, Vass I, Allahverdiyeva Y. 2014. Flavodiiron protein Flv2/Flv4-related photoprotective mechanism dissipates excitation pressure of PSII in cooperation with phycobilisomes in cyanobacteria. Plant Physiology 164, 805–818.

Bezy RP, Wiltbank L, and Kehoe DM. 2011. Light-dependent attenuation of phycoerythrin gene expression reveals convergent evolution of green light sensing in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 108, 18542–18547.

Bode R, Ivanov AG, Hüner NP. 2016. Global transcriptome analyses provide evidence that chloroplast redox state contributes to intracellular as well as long-distance signaling in response to stress and acclimation in Arabidopsis. Photosynthesis Research 128, 287–312.

Boehm M, Yu J, Krynicka V, Barker M, Tichy M, Komenda J, Nixon PJ, and Nield J. 2012. Subunit organization of a *Synechocystis* hetero-oligomeric thylakoid FtsH complex involved in photosystem II repair. Plant Cell 24, 3669–3683.

Bogorad L, and Park YM. 1999. Photomovement of the gliding cyanobacterium *Synechocystis* sp. PCC 6803. Photochemisty and Photobiolology 70, 95–102.

Bombar D, Heller P, Sanchez-Baracaldo P, Cater BJ, Zehr JP. 2014. Comparative genomics reveals surprising divergence of two closely related strains of uncultivated UCYN-A cyanobacteria. ISME Journal 8, 2530–2542.

Boulay C, Wilson A, D'Haene S, Kirilovsky D. 2010. Identification of a protein required for recovery of full antenna capacity in OCP-related photoprotective mechanism in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 107, 11620–11625.

Calzadilla PI, Zhan J, Sétif P, Lemaire C, Solymosi D, Battchikova N, Wang Q, Kirilovsky D. 2019. The cytochrome $b_6f$ complex is not involved in cyanobacterial state transitions. Plant Cell 31, 911–931.

Campbell D, Hurry V, Clarke AK, Gustafsson P, Oquist G. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiology and Molecular Biology Reviews 62, 667–683.

Campbell EL. 2015. Genetic analysis reveals the identity of the photoreceptor for phototaxis in hormogonium filaments of *Nostoc punctiforme*. Journal of Bacteriology 197, 782–791.

Capra EJ, Laub MT. 2012. Evolution of two-component signal transduction systems. Annual Review of Microbiology 66, 325–347.

Chamot D, Colvin KR, Kujat-Choy SL, Owttrim GW. 2005. RNA structural rearrangement via unwinding and annealing by the cyanobacterial RNA helicase, CrhR. Journal of Biological Chemistry 280, 2036–2044.

Chen W, Fang L, Huang X, Ge H, Wang J, Wang X, Zhang Y, Sui N, Xu W, and Wang Y. 2018. Systematic identification of light-regulated cold-responsive proteome in a model cyanobacterium. Journal of Proteomics 179, 100–109.

Chitnis VP, Xu Q, Yu L, Golbeck JH, Nakamoto H, Xie DL, Chitnis PR. 1993. Targeted inactivation of the gene *psaL* encoding a subunit of photosystem I of the cyanobacterium *Synechocystis* sp. PCC 6803. Journal of Biological Chemistry 268, 11678–11684.

Conradi FD, Zhou RQ, Oeser S, Schuergers N, Wilde A, and Mullineaux CW. 2019. Factors controlling floc formation and structure in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Journal of Bacteriology 201, 00344-19.

Cordin O, Tanner NK, Doère M, Linder P, Banroques J. 2004. The newly discovered Q motif of DEAD-box RNA helicases regulates RNA-binding and helicase activity. The EMBO journal 23, 2478-2487.

Dagan T, Roettger M, Stucken K, Landan G, Koch R, Major P, Gould SB, Goremykin VV, Rippka R, Tandeau de Marsac N, Gugger M, Lockhart PJ, Allen JF, Brune I, Maus I, Pühler A, Martin WF. 2013. Genomes of Stigonematalean cyanobacteria (subsection V) and the evolution of oxygenic photosynthesis from prokaryotes to plastids. Genome Biology and Evolution 5, 31–44.

de Porcellinis AJ, Klähn S, Rosgaard L, Kirsch R, Gutekunst K, Georg, J, Hess W. 2016. The Non-Coding RNA Ncr0700/PmgR1 is Required for Photomixotrophic Growth and the Regulation of Glycogen Accumulation in the Cyanobacterium *Synechocystis* sp. PCC 6803. Plant and Cell Physiology 57, 2091–2103.

Derks A, Schaven K, Bruce D. 2015. Diverse mechanisms for photoprotection in photosynthesis. Dynamic regulation of photosystem II excitation in response to rapid environmental change. Biochimica et Biophysica Acta 1847, 468–485.

Dietz KJ. 2015. Efficient high light acclimation involves rapid processes at multiple mechanistic levels. Journal of Experimental Botany 66, 2401–2414.

Eaton SL, Roche SL, Llavero Hurtado M, Oldknow KJ, Farquharson C, Gillingwater TH, Wishart TM. 2013. Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. PLoS ONE 8, e72457.

El Bissati K, Kirilovsky D. 2001. Regulation of *psbA* and *psaE* expression by light quality in *Synechocystis* species PCC 6803. A redox control mechanism. Plant Physiology 125, 1988–2000.

Enami I, Okumura A, Nagao R, Suzuki T, Iwai M, Shen J-R. 2008. Structures and functions of the extrinsic proteins of photosystem II from different species. Photosynthesis Research 98, 349–363.

Ermakova-Gerdes S, Vermaas W. 1998. Mobility of the primary electron-accepting plastoquinone QA of photosystem II in a *Synechocystis* sp. PCC 6803 strain carrying mutations in the D2 protein. Biochemistry 37, 11569-11578.

Espinosa, J, Boyd JS, Cantos R, Salinas P, Golden SS, Contreras A. 2015. Crosstalk and regulatory interactions between the essential response regulator RpaB and cyanobacterial circadian clock output. Proceedings of the National Academy of Sciences of the United States of America 112, 7.

Fairman-Williams, ME, Guenther UP, Jankowsky E. 2010. SF1 and SF2 helicases: family matters. Current opinion in structural biology, 20, 313-324.

Fan DY, Jia H, Barber J, Chow WS. 2009. Novel effects of methyl viologen on photosystem II function in spinach leaves. European Biophysics Journal 39, 191–199.

Fleming KE, O'Shea EK. 2018. An RpaA-Dependent Sigma Factor Cascade Sets the Timing of Circadian Transcriptional Rhythms in *Synechococcus elongatus*. Cell Reports 25, 2937–2945

Fu HY, Picot D, Choquet Y, Longatte G, Sayegh A, Delacotte J, Wollman FA. 2017. Redesigning the $Q_A$ binding site of Photosystem II allows reduction of exogenous quinones. Nature communications 8, 15274.

Fufezan C, Gross CM, Sjödin M, Rutherford AW, Krieger-Liszkay A, Kirilovsky D. 2007. Influence of the redox potential of the primary quinone electron acceptor on photoinhibition in photosystem II. Journal of Biological Chemistry 282, 12492-12502.

Fujisawa T, Masuda, S. 2018. Light-induced chromophore and protein responses and mechanical signal transduction of BLUF proteins. Biophysics Reviews 10, 327–337.

García-Domínguez M, Muro-Pastor MI, Reyes JC, Florencio FJ. 2000. Light-dependent regulation of cyanobacterial phytochrome expression. Journal of Bacteriology, 182, 38-44.

Ge H. 2017 Translating Divergent Environmental Stresses into a Common Proteome Response through the Histidine Kinase 33 (Hik33) in a Model Cyanobacterium. 2017. Journal of Proteomics 16, 1258–1274.

Genty B, Briantais J-M, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochemica et Biophysica Acta 990, 87–92.

Georg J, Rosana ARR, Chamot D, Migur A, Hess WR, Owttrim GW. 2019. Inactivation of the RNA helicase CrhR impacts a specific subset of the transcriptome in the cyanobacterium *Synechocystis* sp. PCC 6803. RNA Biology 16, 1205–1214.

Govindjee and Shevela D. 2011. Adventures with cyanobacteria: a personal perspective. Frontiers in Plant Science 2, 28.

Grossman AR, Schaefer MR, Chiang GG, Collier JL. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiology Reviews. 57, 725– 749.

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A, Saenger W. 2009. Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. Nature Structural & Molecular Biology 16, 334–342.

Gutu A, Kehoe DM. Emerging Perspectives on the Mechanisms, Regulation, and Distribution of Light Color Acclimation in Cyanobacteria. 2011. Nature Reviews 5, 1–13.

Gutu A, Nesbit AD, Alverson AJ, Palmer JD, Kehoe DM. 2013. Unique role for translation initiation factor 3 in the light color regulation of photosynthetic gene expression. Proceedings of the National Academy of Sciences of the United States of America USA 110, 16253–16258.

Hakkila K, Antal T, Rehman AU, Kurkela J, Wada H, Vass I, Tyystjärvi E, Tyystjärvi T. 2014. Oxidative stress and photoinhibition can be separated in the cyanobacterium *Synechocystis* sp. PCC 6803. Biochimica et Biophysica Acta 1837, 217–225.

Hanaoka M, K Tanaka. 2008. Dynamics of RpaB-promoter interaction during high light stress, revealed by chromatin immunoprecipitation (ChIP) analysis in *Synechococcus elongatus* PCC 7942. Plant Journal 56, 327–335.

Henriques R, Jang I, Chua N. Regulated proteolysis in light-related signaling pathways. 2009. Current Opinions in Plant Biology 12, 49–56.

Hihara Y, Sonoike K, Kanehisa M, Ikeuchi M. 2003. DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. Journal of bacteriology 183, 1719-1725.

Hirano M, Satoh K, Katoh, S. 1980. Plastoquinone as a common link between photosynthesis and respiration in blue-green alga. Photosynthesis Research 1, 149–162.

Howitt CA, and Vermaas WFJ. 1998. Quinol and cytochrome oxidases in the cyanobacterium *Synechocystis* sp. PCC 6803. Biochemistry. 37, 17944–17951.

Howitt CA, Cooley JW, Wiskich JT, Vermaas WF. 2001. A strain of *Synechocystis* sp. PCC 6803 without photosynthetic oxygen evolution and respiratory oxygen consumption: implications for the study of cyclic photosynthetic electron transport. Planta 214, 46–56.

Hsiao HY, He Q, Van Waasbergen LG, Grossman AR. 2004. Control of photosynthetic and high-light-responsive genes by the histidine kinase DspA: negative and positive regulation and interactions between signal transduction pathways. Journal of bacteriology 186, 3882-3888.

Huang JY, Chiu YF, Ortega JM, Wang HT, Tseng TS, Ke S, Chu HA. 2016. Mutations of Cytochrome b 559 and PsbJ on and near the QC Site in Photosystem II Influence the Regulation of Short-Term Light Response and Photosynthetic Growth of the Cyanobacterium *Synechocystis* sp. PCC 6803. Biochemistry 55, 2214-2226.

Hubschmann T, Yamamoto H, Gieler T, Murata N, Borner T. 2005. Red and far- red light alter the transcript profile in the cyanobacterium *Synechocystis* sp PCC 6803: impact of cyanobacterial phytochromes. FEBS Letters 579, 1613–1618.

Hurt E and Hauska G. 1981. A cytochrome $b_6f$ complex of five polypeptides with plastoquinol-plastocyanin-oxidoreductase activity from spinach chloroplasts. European Journal of Biochemistry 117, 591–595.

Ibrahim IM, Puthiyaveetil S, Allen JF. 2016. A two-component regulatory system in transcriptional control of photosystem stoichiometry: Redox-dependent and sodium ion-dependent phosphoryl transfer from cyanobacterial histidine kinase Hik2 to response regulators Rre1 and RppA. Frontiers in Plant Science 12, 196–212.

Iijima H, Shirai T, Okamoto M, Kondo A, Hirai MY, Osanai T. 2015. Changes in primary metabolism under light and dark conditions in response to overproduction of a response regulator RpaA in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, Frontiers in Microbiology 6, 888.

Ivleva NB, Gao T, LiWang AC, Golden SS. 2006. Quinone sensing by the circadian input kinase of the cyanobacterial circadian clock. Proceedings of the National Academy of Sciences of the United States of America USA 103, 17468–17473.

Jarmoskaite I, Russell R. 2014. RNA helicase proteins as chaperones and remodelers. Annual review of biochemistry 83, 697-725.

Joshua S, Mullineaux CW. 2004. Phycobilisome diffusion is required for light-state transitions in cyanobacteria. Plant Physiolology 135, 2112–2119.

Jung K, Fried L, Behr S, Heermann R. 2012. Histidine kinases and response regulators in networks. Current Opinion in Microbiology 15, 118–124.

Kaczmarzyk D, Anfelt J, Särnegrim A, Hudson EP. 2014. Overexpression of sigma factor SigB improves temperature and butanol tolerance of *Synechocystis* sp. PCC6803. Journal of Biotechnology, 182, 54–60.

Kadowaki T, Nagayama R, Georg J, Nishiyama Y, Wilde A, Hess WR, Hihara Y. 2016. A Feed-Forward Loop Consisting of the Response Regulator RpaB and the Small RNA PsrR1 Controls Light Acclimation of Photosystem I Gene Expression in the Cyanobacterium *Synechocystis* sp. PCC 6803. Plant and Cell Physiology, 57, 813–823.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Research 3,109–136.

Kanesaki Y, Yamamoto H, Paithoonrangsarid K, Shoumskaya M, Suzuki I, Hayashi H, Murata N. 2007. Histidine kinases play important roles in the perception and signal transduction of hydrogen peroxide in the cyanobacterium, *Synechocystis* sp. PCC 6803. Plant Journal 49, 313–324.

Kappell AD, van Waasbergen LG. 2007. The response regulator RpaB binds the high light regulatory 1 sequence upstream of the high-light-inducible *hliB* gene from the cyanobacterium *Synechocystis* PCC 6803. Archive of Microbiology 187, 337–342.

Kerfeld CA, Kirilovsky, D. 2011. Photoprotection in cyanobacteria: The orange carotenoid protein and energy dissipation. Bioenergetic Processes of Cyanobacteria 312, 395-421.

Khorobrykh S, Tsurumaki T, Tanaka K, Tyystjärvi T, Tyystjärvi E. 2019. Measurement of the redox state of the plastoquinone pool in cyanobacteria. FEBS Letters 33, 1233–9.

Kim YI, Vinyard DJ, Ananyev GM, Dismukes GC, Golden SS. 2012. Oxidized quinones signal onset of darkness directly to the cyanobacterial circadian oscillator. Proceedings of

the National Academy of Sciences of the United States of America USA 109, 17765–17769.

Kinkel TL, Roux CM, Dunman PM, Fang FC. 2013. The Staphylococcus aureus SrrAB two-component system promotes resistance to nitrosative stress and hypoxia. MBio 4, e00696–13.

Kobayashi I, Watanabe S, Kanesaki Y, Shimada T, Yoshikawa H, Tanaka K. 2017. Conserved two-component Hik34-Rre1 module directly activates heat-stress inducible transcription of major chaperone and other genes in *Synechococcus elongatus* PCC 7942. Molecular Microbiology 104, 260-277

Köbler C, Schultz SJ, Kopp D, Voigt K, Wilde A. 2018. The role of the *Synechocystis* sp. PCC 6803 homolog of the circadian clock output regulator RpaA in day- night transitions. Molecular Microbiology 110, 847–861.

Komenda J, Barber J. 1995. Comparison of psbO and psbH deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the QB site and dependent on protein synthesis. Biochemistry, 34, 9625–9631.

Kotajima T, Shiraiwa Y, Suzuki I. 2014. Functional analysis of the N-terminal region of an essential histidine kinase, Hik2, in the cyanobacterium *Synechocystis* sp. PCC 6803. FEMS Microbiology Letters 351, 88–94.

Kujat SL, Owttrim GW. 2000. Redox-Regulated RNA Helicase Expression. Plant Physiology 124, 703-714.

Kuwahara A, Arisaka S, Takeya M, Iijima H, Hirai MY, Osanai T. 2015. Modification of photosynthetic electron transport and amino acid levels by overexpression of a circadian-related histidine kinase *hik8* in *Synechocystis* sp. PCC 6803. Frontiers in Microbiology 6, 637–9.

Lau N, Matsui M, Abduallah AA. 2015. Cyanobacteria: Photoautotrophic Microbial Factories for the Sustainable Synthesis of Industrial Products. BioMed Research International 8, 754934.

Lea-Smith DJ, Ross N, Zori M, Bendall DS, Dennis JS, Scott SA, Smith AG, Howe CJ. 2013. Thylakoid terminal oxidases are essential for the cyanobacterium *Synechocystis* sp. PCC 6803 to survive rapidly changing light intensities. Plant Physiology 162, 484–495.

Lee JH, Jung JH, Park CM. 2017. Light inhibits COP1-mediated degradation of ICE transcription factors to induce stomatal development in Arabidopsis. Plant Cell. 29, 2817–2830.

Li H, Sherman LA. 2000. A redox-responsive regulator of photosynthesis gene expression in the cyanobacterium *Synechocystis* sp. Strain PCC 6803. Journal of Bacteriology 182, 4268–4277.

Linder P, Owttrim GW. 2009. Plant RNA helicases: linking aberrant and silencing RNA. Trends in Plant Science 14, 344–352.

Linder P, Jankowsky E. 2011. From unwinding to clamping — the DEAD box RNA helicase family. Nature Reviews Molecular Cell Biology, 12, 505–516.

Locato V, Cimini S, De Gara, L. 2018. ROS and redox balance as multifaceted players of cross-tolerance: epigenetic and retrograde control of gene expression. Journal of Experimental Botany. 69, 3373–3391.

Luimstra VM, Schuurmans JM, Verschoor AM, Hellingwerf KJ, Huisman J, Matthijs HCP. 2018. Blue light reduces photosynthetic efficiency of cyanobacteria through an imbalance between photosystems I and II. Photosynthesis Reseach. 138, 77–189.

Luo H, Jackson SA, Fagerlund RD, Summerfield TC, Eaton-Rye JJ. 2014. The importance of the hydrophilic region of PsbL for the plastoquinone electron acceptor complex of Photosystem II. Biochimica et Biophysica Actaf-Bioenergetics, 1837, 1435-1446.

Ma W, Deng Y, Mi H. 2008. Redox of plastoquinone pool regulates the expression and activity of NADPH dehydrogenase supercomplex in *Synechocystis* sp. strain PCC 6803. Current Opinions in Microbiology 56, 189–193.

MacColl R. 1998. Cyanobacterial phycobilisomes. Journal of Structural Biology 124, 311–334

Maksimov EG, Mironov KS, Trofimova MA, Nechaeva NL, Todorenko DA, Klementiev KE, Tsoraev GV, Tyutyaev EV, Zorina AA, Feduraev PV, Allakhverdiev SI, Paschenko VZ, Los DA. 2017. Membrane fluidity controls redox-regulated cold stress responses in cyanobacteria. Photosynthesis Research 133, 215–223.

Mao M. 2002. The redox state of plastoquinone pool regulates state transitions via cytochrome b. FEBS Letters. 519, 82–86.

Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N. 2003. Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis* sp. PCC 6803. Proceedings of the National Academy of Sciences of the United States of America USA 100, 9061–9066.

Markson JS, Piechura JR, Puszynska AM, O'Shea EK. 2013. Circadian Control of Global Gene Expression by the Cyanobacterial Master Regulator RpaA. Cell 155, 1396–1408.

Martin R, Straub AU, Doebele C, Bohnsack MT. 2013. DExD/H-box RNA helicases in ribosome biogenesis. RNA biology 10, 4-18.

Mikami K, Kanesaki Y, Suzuki I, Murata N. 2002. The histidine kinase Hik33 perceives osmotic stress and cold stress in *Synechocystis* sp PCC 6803. Molecular Microbiology 46, 905–915.

Mironov KS, Sidorov RA, Trofimova MS, Bedbenov VS, Tsydendambaev VD, Allakhverdiev SI., Los DA. 2012. Light-dependent cold-induced fatty acid unsaturation, changes in membrane fluidity, and alterations in gene expression in *Synechocystis*. Biochimica et Biophysica Acta 1817, 1352–1359.

Mironov KS, Sidorov RA, Kreslavski VD, Bedbenov VS, Tsydendambaev VD, and Los DA. 2014. Cold-induced gene expression and ω(3) fatty acid unsaturation is controlled by red light in *Synechocystis*. Journal of Photochemistry and Photobiology 137, 84–88.

Misumi M, Katoh H, Tomo T, Sonoike K. 2016. Relationship Between Photochemical Quenching and Non-Photochemical Quenching in Six Species of Cyanobacteria Reveals Species Difference in Redox State and Species Commonality in Energy Dissipation. Plant and Cell Physiology 57, 1510–1517.

Mizuno T, Kaneko T, Tabata S. 1996. Compilation of All Genes Encoding Bacterial Two-component Signal Transducers in the Genome of the Cyanobacterium, *Synechocystis* sp. Strain PCC 6803. DNA Research 3, 407-414.

Mohamed A, Jansson C. 1991. Photosynthetic electron transport controls degradation but not production of psbA transcripts in the cyanobacterium *Synechocystis* 6803. Plant molecular biology 16, 891-897.

Montgomery BL. 2007. Sensing the light: photoreceptive systems and signal transduction in cyanobacteria. Molecular Microbiology 64, 16–27.

Mullineaux CW, Allen JF. 1986. The state 2 transition in the cyanobacterium *Synechococcus* 6301 can be driven by respiratory electron flow into the plastoquinone pool. FEBS Letters. 205, 155-160.

Mullineaux CW, Allen JF. 1990. State 1-State 2 transitions in the cyanobacterium *Synechococcus* 6301 are controlled by the redox state of electron carriers between Photosystems I and II. Photosynthesis Research 23, 297–311.

Mullineaux CW. 2013. Co-existence of photosynthetic and respiratory activities in cyanobacterial thylakoid membranes. Biochimica et Biophysica Acta 1837, 503–511.

Mullineaux CW, Emlyn-Jones D. 2005. State transitions: an example of acclimation to low-light stress. Journal of Experimental Botany 56, 389–393.

Murata N, Suzuki I. 2006. Exploitation of genomic sequences in a systematic analysis to access how cyanobacteria sense environmental stress. Journal of Experimental Botany 57, 235–247.

Nelson N, Ben-Shem A. 2004. The complex architecture of oxygenic photosynthesis. Nature Reviews 5, 971–982.

Nishiyama Y, Yamamoto H, Allakhverdiev S, Inaba M, Yokota A, Murata N. 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. EMBO Journal 20, 5587–5594.

Nixon P, Michoux F, Yu J, Boehm M, Komenda J. 2010. Recent advances in understanding the assembly and repair of photosystem II. Annals of Botany 106, 1–16.

Ogawa T, Harada T, Ozaki H, Sonoike K. 2013. Disruption of the ndhF1 gene affects Chl fluorescence through state transition in the Cyanobacterium *Synechocystis* sp. PCC 6803, resulting in apparent high efficiency of photosynthesis. Plant And Cell Physiology 54, 1164–1171.

Ogawa T, Misumi M, Sonoike K. 2017. Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions. Photosynthesis Research 133, 63–73.

Osanai T, Kuwahara A, Iijima H, Toyooka K, Sato M, Tanaka K, Ikeuchi M, Saito K, Hirai MY. 2013. Pleiotropic effect of sigE over-expression on cell morphology, photosynthesis and hydrogen production in *Synechocystis* sp. PCC 6803. Plant Journal 76, 456–465.

Osanai T, Shirai T, Iijima H, Kuwahara A, Suzuki I, Kondo A, Hirai MY. 2015. Alteration of cyanobacterial sugar and amino acid metabolism by overexpression hik8, encoding a KaiC-associated histidine kinase. Environmental Microbiology 17, 2430–2440.

Owttrim, G.W. 2012. RNA helicases in cyanobacteria: biochemical and molecular approaches. Methods in Enzymology 511, 385–403.

Owttrim, G.W. 2013. RNA helicases: diverse roles in prokaryotic response to abiotic stress. RNA Biology 10, 96–110.

Oxborough K, Baker NR. 1997. Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of qP and Fv-Fm; without measuring Fo-. Photosynthesis Research 54, 135–142.

Paik I, Yang S, Choi, G. 2012. Phytochrome regulates translation of mRNA in the cytosol. Proceedings of the National Academy of Sciences of the United States of America USA 109, 335–1340.

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I, et al. 2004. Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. Journal of Biological Chemistry 279, 53078–53086.

Park E, Kim Y, Choi, G. 2018. Phytochrome B Requires PIF Degradation and Sequestration to Induce Light Responses across a Wide Range of Light Conditions. Plant Cell 30, 1277–1292.

Piechura JR, Amarnath K, O'Shea EK. 2017. Natural changes in light interact with circadian regulation at promoters to control gene expression in cyanobacteria, eLife. 6, e32032.

Pinto F, Pacheco CC, Ferreira D, Moradas-Ferreira P, Tamagnini P. 2012. Selection of suitable reference genes for RT-qPCR analyses in cyanobacteria. PLoS ONE 4, e34983.

Plohnke N, Seidel T, Kahmann U, Rogner M, Schneider D, Rexroth S. 2015. The proteome and lipidome of *Synechocystis* sp. PCC 6803 cells grown under light-activated heterotrophic conditions. Molecular Cell Proteomics 14, 572–584.

Prakash JS, Krishna PS., Sirisha K, Kanesaki Y, Suzuki I, Shivaji S, Murata, N. 2010. An RNA helicase, CrhR, regulates the low-temperature-inducible expression of heat-shock genes groES, groEL1 and groEL2 in *Synechocystis* sp. PCC 6803. Microbiology 156, 442-451

Preston C, Critchley C. 1988. Interaction of electron acceptors with thylakoids from halophytic and non-halophytic species. Photosynthesis Research 16, 187–202.

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF. 2008. The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. Proceedings of the National Academy of Sciences of the United States of America USA 105, 10061–10066.

Qi Q, Hao M, Ng WO, Slater SC, Baszis SR, Weiss JD, Valentin HE. 2005. Application of the *Synechococcus* nirA Promoter to Establish an Inducible Expression System for Engineering the *Synechocystis* Tocopherol Pathway. Applied and Environmental Microbiology 71, 5678–5684.

Rastogi RP, Singh SP, Häder DP, Sinha RP. 2010. Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate in the cyanobacterium *Anabaena variabilis* PCC 7937. Biochemical and Biophysical Research Communications 397 603–607.

Raven JA, Allen JF. 2003. Genomics and chloroplast evolution: what did cyanobacteria do for plants? Genome Biology 4, 209.

Riediger M. 2019. Biocomputational Analyses and Experimental Validation Identify the Regulon Controlled by the Redox- Responsive Transcription Factor RpaB. 2019. iScience 15, 316–331.

Ritter SPA, Lewis AC, Vincent S., Lo LL, Cunha APA, Chamot D, Ensminger I, Espie GS. Owttrim GW. 2019. Evidence for convergent sensing of multiple abiotic stresses in cyanobacteria. Biochemica et Biophysica Acta. 1864, 129462.

Roberts AG, Kramer DM. 2001. Inhibitor "double occupancy" in the Q(o) pocket of the chloroplast cytochrome $b_6f$ complex. Biochemistry 40, 13407–13412.

Rocak S, Linder P. 2004. DEAD-box proteins: the driving forces behind RNA metabolism. Nature reviews Molecular Cell Biology 5, 232.

Rockwell NC, Lagarias JC. 2017. Phytochrome diversification in cyanobacteria and eukaryotic algae. Current Opinions in Plant Biology 37, 87–93.

Rosana AR, Chamot D, Owttrim GW. 2012a. Autoregulation of RNA helicase expression in response to temperature stress in *Synechocystis* sp. PCC 6803. PLoS ONE 7, e48683.

Rosana AR, Ventakesh M, Chamot D, Patterson-Fortin LM, Tarassova O, Espie GS, Owttrim GW. 2012b. Inactivation of a low temperature-induced RNA helicase in *Synechocystis* sp. PCC 6803: physiological and morphological consequences. Plant and Cell Physiology 53, 646–658.

Rosana A.R, Whitford DS, Fahlman RP, and Owttrim GW. 2016. Cyanobacterial RNA helicase CrhR localizes to the thylakoid membrane region and cosediments with degradosome and polysome complexes in *Synechocystis* sp. strain PCC 6803. Journal of Bacteriology 198, 2089–2099.

Rowland MA, Deeds EJ. 2014. Crosstalk and the evolution of specificity in two-component signaling. Proceedings of the National Academy of Sciences of the United States of America USA 111, 5550–5555.

Rowland JG, Simon WJ, Prakash JS, Slabas AR. 2011. Proteomics reveals a role for the RNA helicase crhR in the modulation of multiple metabolic pathways during cold acclimation of *Synechocystis* sp. PCC6803. Journal of proteome research, 10, 3674-3689.

Satoh K, Koike H, Ichimura T, Katoh S. 1992. Binding affinities of benzoquinones to the QB site of photosystem II in *Synechococcus* oxygen-evolving preparation. Biochimica et Biophysica Acta 1102, 5-52

Schneider CA, Rasband WS, Eliceiri KW. 2012. "NIH Image to ImageJ: 25 years of image analysis", Nature Methods 9, 671–675.

Schreiber U, Endo T, Mi H, Asada K 1995. Quenching analysis of chlorophyll fluorescence by the saturation pulse method: particular aspects relating to the study of eukaryotic algae and cyanobacteria. Plant Cell Physiology 36, 873–882.

Schreiber U. 1986. Detection of rapid induction kinetics with a new type of high frequency modulated chlorophyll fluorometer. Photosynthesis Research 9, 261–272.

Schuurmans RM, Matthijs JC, Hellingwerf KJ. 2017. Transition from exponential to linear photoautotrophic growth changes the physiology of *Synechocystis* sp. PCC 6803. Photosynthesis Research 132, 69–82.

Schuurmans RM, Schuurmans JM, Bekker M, Kromkamp JC, Matthijs HCP, Hellingwerf KJ. 2014. The Redox Potential of the Plastoquinone Pool of the Cyanobacterium *Synechocystis* Species Strain PCC 6803 Is under Strict Homeostatic Control. Plant Physiology 165, 463–475.

Schuurmans RM, van Alphen P, Schuurmans JM, Matthijs HC, Hellingwerf KJ. 2015. Comparison of the Photosynthetic Yield of Cyanobacteria and Green Algae: Different Methods Give Different Answers. PLoS ONE 10, e0139061.

Seino Y, Takahashi T, Hihara, Y. 2009. The Response Regulator RpaB Binds to the Upstream Element of Photosystem I Genes To Work for Positive Regulation under Low-Light Conditions in *Synechocystis* sp. Strain PCC 6803. Journal of Bacteriology 191, 1581–1586.

Sétif P. 2015. Electron-transfer kinetics in cyanobacterial cells: methyl viologen is a poor inhibitor of linear electron flow. Biochimica et Biophysica Acta 1847, 212–222.

Shen S, Fang FC. 2012. Integrated stress responses in Salmonella. International Journal of Food Microbiology 152, 75–78.

Shevela D, Messinger J 2012. Probing the turnover efficiency of photo- system II membrane fragments with different electron acceptors. Biochimica et Biophysica Acta 1817, 208–1212

Shimura Y, Shiraiwa Y, Suzuki I. 2012. Characterization of the subdomains in the N-terminal region of histidine kinase Hik33 in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant and Cell Physiology 53, 1255–1266.

Shoumskaya MA, Paithoonrangsarid K, Kanesaki Y, Los DA, Zinchenko VV, Tanticharoen M, Suzuki I, Murata N. 2005. Identical Hik-Rre systems are involved in perception and transduction of salt signals and hyperosmotic signals but regulate the expression of individual genes to different extents in *Synechocystis*. Journal of Biological Chemistry 280, 21531-8.

Suzuki I, Murata N. 2005.Identical Hik-Rre systems are involved in perception and transduction of salt signals and hyperosmotic signals but regulate the expression of individual genes to different extents in *Synechocystis*. Journal of Biological Chemistry 280, 21531-8.

Sinetova MA, Los DS. 2016. New insights in cyanobacterial cold stress responses: Genes, sensors, and molecular triggers. Biochimica et Biophysica Acta 1863, 2391–2403.

Singh AK, Sherman LA. 2005. Pleiotropic effect of a histidine kinase on carbohydrate metabolism in *Synechocystis* sp. strain PCC 6803 and its requirement for heterotrophic growth. Journal of Bacteriology 187, 2368–2376.

Sireesha K, Radharani B, Krishna PS, Sreedhar N, Subramanyam R, Mohanty P, Prakash JS. 2012. RNA helicase, CrhR is indispensable for the energy redistribution and the regulation of photosystem stoichiometry at low temperature in *Synechocystis* sp. PCC6803. Biochimica et Biophysica Acta 1817, 1525–1536.

Slabas AR, Suzuki I, Murata N, Simon WJ, Hall JJ. 2006. Proteomic analysis of the heat shock response in *Synechocystis* PCC6803 and a thermally tolerant knockout strain lacking the histidine kinase 34 gene. Proteomics 6, 845–864.

Suzuki I, Los DA, Kanesaki Y, Mikami K, Murata N. 2000. The pathway for perception and transduction of low-temperature signals in Synechocystis. The EMBO Journal. 19, 1327-1334.

Suzuki I. 2005. The Histidine Kinase Hik34 Is Involved in Thermotolerance by Regulating the Expression of Heat Shock Genes in *Synechocystis*. 2005. Plant Physiology 138, 1409–1421.

Suzuki I, Kanesaki Y, Hayashi H, Hall JJ, Simon WJ, Slabas AR, Murata N. 2005. The histidine kinase Hik34 is involved in thermotolerance by regulating the expression of heat shock genes in *Synechocystis*. Plant Physiology 138, 1409–1421.

Suzuki I, Kanesaki Y, Mikami K, Kanehisa M, Murata N. 2001. Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. Molecular Microbiology 40, 235–244.

Suzuki I, Los DA, Kanesaki Y, Mikami K, Murata N. 2000. The pathway for perception and transduction of low-temperature signals in *Synechocystis*. The EMBO Journal. 196, 1327-1334.

Tabei I. 2012. Two Regulatory Networks Mediated by Light and Glucose Involved in Glycolytic Gene Expression in Cyanobacteria. 2012. Journal of Proteomics 53, 1720–1727.

Takahashi T, Nakai N, Muramatsu M, Hihara Y. 2010. Role of Multiple HLR1 Sequences in the Regulation of the Dual Promoters of the *psaAB* Genes in *Synechocystis* sp. PCC 6803. 2010. Molecular Microbiology. 192, 4031–4036.

Takai N. 2006. A KaiC-associating SasA–RpaA two-component regulatory system as a major circadian timing mediator in cyanobacteria. 2006. Proceedings of the National Academy of Sciences of the United States of America 103, 2109-12114.

Tarassova OS, Chamot D. Owttrim GW. 2014. Conditional, temperature-induced proteolytic regulation of cyanobacterial RNA helicase expression. Journal of Bacteriology 193, 1560– 1568.

Trebitsh T, Danon A. 2001. Translation of chloroplast psbA mRNA is regulated by signals initiated by both photosystems II and I. Proceedings of the National Academy of Sciences of the United States of America USA 98, 12289–12294.

Trebst A. 1980. Inhibitors in electron flow: tools for the functional and structural localization of carriers and energy conservation sites. Methods in Enzymology 69, 675–715.

Tu CJ, Shrager J, Burnap RL, Postier BL, Grossman AR. 2004. Consequences of a deletion in dspA on transcript accumulation in *Synechocystis* sp. strain PCC6803. Journal of Bacteriology 186, 3889–3902.

Tullberg A, Alexciev K, Pfannschmidt T, Allen JF. 2000. Photosynthetic electron flow regulates transcription of the *psaB* gene in pea (Pisum sativum L.) chloroplasts through the redox state of the plastoquinone pool. Plant and Cell Physiology 41, 1045–1054.

Tyystjärvi T, Herranen M, Aro EM. 2001. Regulation of translation elongation in cyanobacteria: membrane targeting of the ribosome nascent-chain complexes controls the synthesis of D1 protein. Molecular Microbiology 240, 476–484.

Umena Y, Kawakami K, Shen JR, Kamiya N. 2011. Crystal structure of oxygen evolving photosystem II at a resolution of 1.9 A. Nature. 473, 55-60.

van Beilen WA, Hellingwerf KJ. 2016. All Three Endogenous Quinone Species of Escherichia coli Are Involved in Controlling the Activity of the Aerobic/Anaerobic Response Regulator ArcA. Frontiers in Microbiology 7, 1402–11.

Vidal R, Lopez-Maury L, Guerrero MG, Florencio FJ. 2009. Characterization of an Alcohol Dehydrogenase from the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 That Responds to Environmental Stress Conditions via the Hik34-Rre1 Two-Component System. Journal of Bacteriology 191, 4383–4391.

Vinnemeier J, Hagemann M. 1999. Identification of salt-regulated genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803 by subtractive RNA hybridization. Archives of Microbiology 172, 377–386.

Wardman P. 2007. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls and prospects. Free Radical Biology and Medicine 43, 995–1022.

Welkie DG, Rubin BE, Chang YG, Diamond S, Rifkin SA, LiWang A, Golden SS. 2018. Genome-wide fitness assessment during diurnal growth reveals an expanded role of the cyanobacterial circadian clock protein KaiA. Proceedings of the National Academy of Sciences of the United States of America USA 115, 7174–E7183.

Westermark S, Steuer R. 2016. Toward multiscale models of cyanobacterial growth: A modular approach. Frontier in Bioengineering and Biotechnology 4, 95.

Wilde A, Hihara Y. 2016. Transcriptional and posttranscriptional regulation of cyanobacterial photosynthesis. Biochimica et Biophysica Acta 1857, 296–308.

Wilde A, Fiedler B, Borner T. 2002. The cyanobacterial phytochrome Cph2 inhibits phototaxis towards blue light. Molecular Microbiology 44, 981–988.

Wiltbank LB, Kehoe DM. 2016. Two cyanobacterial photoreceptors regulate photosynthetic light harvesting by sensing teal, green, yellow and red light. mBio 7, e02130– 15.

Wiltbank LB, Kehoe DM. 2019. Diverse light responses of cyanobacteria mediated by phytochrome superfamily photoreceptors. Nature Reviews Microbiology 17, 37–50.

Xia Y and Xun L. 2017. Revised Mechanism and Improved Efficiency of the QuikChange Site-Directed Mutagenesis Method. Methods in Molecular Biology 1498, 367-374.

Zhang P Aro EM. 2012. Operon *flv4-flv2* Provides Cyanobacterial Photosystem II with Flexibility of Electron Transfer. Plant Cell 24, 1952–1971.

Zito I. 1999. The $Q_O$ site of cytochrome $b_6f$ complexes controls the activation of the LHCII kinase. EMMO. 18, 2961-2969.

Zubkov MV, Fuchs BM, Tarran GA, Burkill PH, Amann R. 2003. High Rate of Uptake of Organic Nitrogen Compounds by Prochlorococcus Cyanobacteria as a Key to Their Dominance in Oligotrophic Oceanic Waters. Applied and Environmental Microbiology 69, 1299–1304.