ABSTRACT

PHOTOXIDATIVE STRESS RESPONSE IN MESOPHILIC AND PSYCHROPHILIC STRAINS OF CHLAMYDOMONAS RAUDENSIS: A COMPARATIVE STUDY

by Sarah E. Stahl

Chlamydomonas raudensis UWO241 is a model psychrophilic alga with a close mesophilic relative C. raudensis SAG49.72. UWO241 was isolated from the ice-covered Lake Bonney (McMurdo Dry Valleys, Antarctica) and is adapted to low temperatures ($T_{opt}=8^\circ$C), low light and high salinity (700mM NaCl), where SAG49.72 was isolated from a temperate lake in Europe, is salt-sensitive and adapted to moderate temperature and light conditions. These two organisms present a unique opportunity to compare environmental adaptation between two closely related organisms isolated from divergent habitats. In this study we explored the ability of UWO241 and SAG49.72 to acclimate to long- and short-term oxidative stresses. Goals of this thesis were to characterize: (i) long-term stress acclimation and (ii) short-term stress avoidance mechanisms in psychrophilic and mesophilic algae. Under long-term stress UWO241 exhibited enhanced ROS detoxification such as ascorbate peroxidase and glutathione reductase enzyme activity while SAG49.72 exhibited enhanced photochemical modifications. Additionally, UWO241 had limited recovery from short-term high light stress.
PHOTOOXIDATIVE STRESS RESPONSE IN MESOPHILIC AND PSYCHROPHILIC STRAINS OF CHLAMYDOMONAS RAUDENSIS: A COMPARATIVE STUDY

A Thesis

Submitted to the
Faculty of Miami University
in partial fulfillment of
the requirements for the degree of
Masters of Science
Department of Microbiology
by
Sarah E. Stahl
Miami University
Oxford, OH
2014

Advisor________________________________
Dr. Rachael Morgan-Kiss

Reader________________________________
Dr. Mitch Balish

Committee Member____________________________
Dr. Gary Janssen

Committee Member____________________________
Dr. Andor Kiss

Committee Member____________________________
Dr. Natasha Finley
TABLE OF CONTENTS

Chapter One: Introduction ........................................... P. 1-13

Chapter Two: Characterization of long-term stress response mechanisms in psychrophile *C. raudensis* UWO241 and mesophile *C. raudensis* SAG49.72 ........................................... P. 14-50

Chapter Three: Characterization of short-term stress response mechanisms in psychrophile *C. raudensis* UWO241 and mesophile *C. raudensis* SAG49.72 ........................................... P. 51-67

Chapter Four: Conclusion ........................................... P. 68-75

References ............................................................. P. 76-82
LIST OF TABLES

Table 1.1: List of multiple permanent environmental stresses faced by *C. raudensis* UWO241 in its natural habitat and its adaptations to permanent stress. P. 13

Table 2.1: Growth physiology parameters in cultures of UWO241 and SAG49.72 grown under control versus long-term stress conditions. P. 32

Table 2.2: PSI activity determined by rate of oxidation in UWO241 and SAG49.72 acclimated to long-term stress conditions. P. 38

Table 2.3: Summary of Gaussian fitting parameters for the subband decompositions of 77K Chl a fluorescence spectra in UWO241 and SAG49.72 cultures acclimated to control vs. long-term stress conditions. P. 41

Table 2.4: Candidate reference genes and glycerol synthase gene, *Serb*, identified from cDNA sequence library of UWO241. P. 48

Table 2.5. Quantification of total lipid bodies present, average lipid body radius, average lipid body volume and total volume lipid bodies per cell of UWO241 or SAG49.72 acclimated to long-term stress. P. 50
LIST OF FIGURE

CHAPTER ONE

Figure 1.1: A simplified overview of the impact of oxidative stress on the photosynthetic apparatus. P.11

Figure 1.2: The ascorbate-glutathione pathway. P. 12

CHAPTER TWO

Figure 2.1: Long-term stress treatment conditions for UWO241 and SAG49.72. P. 31

Figure 2.2: Maximum growth rates in cultures of UWO241 and SAG49.72 grown under control vs. long-term stress conditions. P. 33

Figure 2.3: Ratios of Chl a/b in cultures of UWO241 and SAG49.72 grown under control vs. long-term stress conditions. P. 34

Figure 2.4: Relative quantification of PsbA and RubisCO (RbcL) protein abundance in UWO241 and SAG49.72 grown under control vs. long-term stress conditions. P.35

Figure 2.5: Effect of long-term stress acclimation on photochemical quenching and energy partitioning of PSII in UWO241 and SAG 49.72. P.36

Figure 2.6: Effect of long-term stress acclimation on photosystem I activity determined by P700 in UWO241 and SAG 49.72. P. 37
Figure 2.7: Effect of long-term stress acclimation on energy partitioning in Photosystem I in UWO241 and SAG 49.72 grown under control vs. stress conditions.

Figure 2.8: 77K fluorescence emission spectra and decomposition in Gaussian subbands of UWO241 and SAG49.72 acclimated to long-term stress treatments.

Figure 2.9: The quantum yield of CEF to LEF in UWO241 and SAG49.72 acclimated to long-term stress treatments.

Figure 2.10: Relative quantification of ascorbate peroxidase protein abundance in UWO241 and SAG49.72 grown under control vs. long-term stress conditions.

Figure 2.11: Representative immunoblot of ascorbate peroxidase isolated from UWO241 and SAG49.72 cultures grown under control conditions.

Figure 2.12: Ascorbate peroxidase activity in UWO241 and SAG49.72 grown under control vs. long-term stress conditions.

Figure 2.13: Total glutathione reductase activity per total protein of UWO241 and SAG49.72 grown under control vs. long-term stress conditions.

Figure 2.14: Expression of \textit{serB} gene quantified by real time quantitative PCR across cultures of \textit{C. raudensis} UWO241 grown under variable salinity.

Figure 2.15: Total lipid bodies in UWO241 and SAG49.72 acclimated to long-term stress conditions.
CHAPTER THREE

Figure 3.1: Determination of sensitivity of UWO241 vs. SAG49.72 algae to short-term photooxidative stress. P. 62

Figure 3.2: Percent photoinhibition in UWO241 and SAG49.72 induced during short-term stress conditions and recovery. P. 63

Figure 3.3: Effect of short-term stress response and recovery on photochemical quenching, qL and energy partitioning of PSII in UWO241 and SAG 49.72. P. 64

Figure 3.4: Relative quantification of PsbA and RbcL protein abundance in UWO241 and SAG49.72 exposed high light and low temperature short-term stress over 1 hour. P. 65

Figure 3.5: Production of reactive oxygen species in UWO241 vs. SAG49.72 during short-term incubation in low temperature or high light stress. P. 66

Figure 3.6: Sensitivity of UWO241 and SAG49.72 to varying concentrations of chemical oxidants that result in production of different ROS molecules. P. 67

CHAPTER FOUR

Figure 4.1: A simplified overview of photooxidative stress response mechanisms in C. raudensis UWO241 and SAG49.72 during photoacclimation to control, high light, low temperature and high salt stress. P. 74- 75
I would like to thank the Department of Microbiology at Miami University and the National Science Foundation (NSF) for supporting my research, which could not have been done without the support and guidance of my advisor, Dr. Rachael Morgan-Kiss. I would like to extend a big thank you to Dr. Morgan-Kiss for all that she does.

I’d like to thank my collaborators Andor Kiss of the CBFG at Miami University for the transcriptome of UWO241 and James Raymond of University of Nevada Las Vegas for glycerol analysis. I also want to thank my committee Dr. Andor Kiss, Dr. Gary Janssen, Dr. Natosha Finley and a special thanks to Dr. Mitch Balish for reading and reviewing my thesis. Additionally I'd like to acknowledge my current and former lab mates for their constant support and assistance: Jenna Dolhi, Amber Teufel, Wei Li, Nicholas Ketchum and special thanks for two undergraduate who collaborated on this research, Justin Prusz and Mark Hahn. Finally, I’d like to thank my family and friends for their love and support.
CHAPTER ONE

INTRODUCTION

1.1 ENVIRONMENTAL STRESS

Since oxygen was introduced into the atmosphere ~2.7 billion years ago, organisms have required the ability to survive oxidative stress (Sirikhachornkit and Niyogi 2010, Singh Gill and Tuteja 2010). Environmental stressors, such as drought, cold temperatures, high light and increasing soil salinity, are the main limiting factors in plant productivity (Allen 1995). Salinization of soil is recognized as a global threat affecting almost 1 billion hectare of cropland worldwide (Ondrasek et al. 2011). The majority of crops are cold sensitive and extreme crop loss can occur due to fluctuating temperatures and longer frost times. Research on cold adapted plants as well as “single-celled plants” (i.e., microalgae) has great potential to help reduce cold stress crop loss. To ultimately improve stress resistance one must characterize the impacts of stress and understand the defense mechanisms across organisms adapted to a broad range of environments (Lyons et al. 1979). Our understanding of oxidative stress response in organisms adapted to cold temperatures as well as additional stressors is currently limited. This study extends our understanding of environmental stress adaptation and oxidative stress response by comparing adaptive strategies to long- and short-term photooxidative stress in a high salt, low light and low temperature-adapted extremophilic algal species with a cold sensitive mesophilic algae relative.

1.2 OXIDATIVE STRESS ON THE PHOTOSYNTHETIC APPARATUS

Light is essential to all photosynthetic organisms; however, exposure to excessive light that is beyond levels needed for energy generation leads to photooxidative stress. In plants and algae, photoinhibition is a feature of photooxidative stress, which is a decrease in photosynthetic performance due to an imbalance between energy produced by photochemical reactions and energy consumed by downstream reactions. If this imbalance is not compensated for through several processes generally termed photostasis, then the organism will experience photoinhibition, which can lead to the production of reactive oxygen species (ROS). ROS can also accumulate in cells when ROS detoxification pathways become overwhelmed. Thus, acclimation to stress and rebalancing of photostasis, as well as induction of ROS defense pathways, are essential for photosynthetic organisms to survive.
Photostasis is the balance between energy absorbed by the photosynthetic apparatus (i.e., at the site of photosystems I and II [PSI and PSII, respectively]) and energy utilized by downstream metabolic processes like carbon fixation (Sirikhachornkit and Niyogi 2010, Takahashi and Murata 2008, Niyogi 2009, Apel and Hirt 2004). Photostasis can be disrupted by both the over-reduction of the plastoquinone (PQ) pool as well as limited regeneration of the electron acceptor NADP⁺, reducing normal electron flow through photosynthesis (Fig. 1.1). Under conditions of environmental stress, for example, high light, PSII becomes over-excited generating excess electrons that cause over reduction of the PQ pool. Once the electron transport chain is over-reduced, additional electrons generated during photochemical reactions will be deposited onto oxygen in the chloroplasts to produce harmful ROS (Fig. 1.1). Thus, any environmental stress that disrupts photostasis will result in over-reduction of the photosynthetic electron transport chain, which may lead to production of ROS.

There are several forms of ROS that are selectively produced in the peroxisomes and chloroplasts. In the chloroplast, two pigment-protein complexes are responsible for production of ROS. PSII directly photoreduces oxygen to singlet oxygen (¹O₂) and PSI electron transport intermediates convert oxygen to superoxide (·O₂⁻) when NADP⁺ availability is limited. Superoxide is further reduced to a less toxic ROS, hydrogen peroxide (H₂O₂) by the enzyme superoxide dismutase (Apel and Hirt 2004, Singh Gill and Tuteja 2010, Ledford et al. 2007, Sirikhachornkit and Niyogi 2010, Forester et al. 2005; Fig. 1.1). Photorespiration can act as an electron sink to prevent photoinhibition but can also be a major source of H₂O₂ production. H₂O₂ is produced in a side reaction in peroxisomes, which occurs as a result of oxygenase activity of the enzyme RubisCO that results in production of glycolate (Apel and Hirt 2004).

Photosynthetic organisms continually produce ROS, which if not detoxified, causes oxidative injury and damage to many essential biological molecules such as proteins, lipids, and nucleic acids (Halliwell et al. 1989, Sirikhachornkit and Niyogi 2010, Singh and Tuteja 2010, Apel and Hirt 2004, Davis et al. 2013). The PQ pool can act as a chloroplast redox sensor to sense and respond to oxidative stress, thus regulating ROS defense mechanisms (Hüner et al. 1998; Fig. 1.1). Additional stressors (e.g. heat, chilling, nutrient deficiency and salinity) exacerbate the potential for ROS production by the photosynthetic apparatus.
1.3 CHLOROPHYLL FLUORESCENCE

Photosynthetic performance in plants and algae can be monitored using the saturating pulse method of room temperature Chlorophyll a (Chl a) fluorescence (Baker 2008). Maximum quantum efficiency of PSII photochemistry ($F_{v}/F_{m}$) is the most common measurement to determine photochemical ability and thus percent photoinhibition (Niyogi 2009). Chl fluorescence can be used to detect modulations in the redox state of the PQ pool as well as energy partitioning (Niyogi 2009). Energy partitioning measurements monitor energy going into photosynthesis ($\Phi_{PSII}/\Phi_{PSI}$) vs. energy dissipated through non-photochemical quenching ($\Phi_{NPQ}$) and non-inducible photochemical quenching ($\Phi_{NO}$), both of which are primarily lost as heat. Low temperature 77K chlorophyll fluorescence can also be used to monitor energy distribution between major pigment-protein complexes, including light harvesting complex (LHCII), PSII and PSI.

The relative oxidized states of QA, the first stable quinone electron acceptor of PSII, can be measured via qL. This measurement reflects the redox state of the plastoquinone pool and the intersystem electron transport chain (Szyszka et al. 2007, Huñer et al. 1998, Kramer et al. 2004, Gray et al. 1996). When qL=1, PSII is fully oxidized and therefore all PSII reaction centers are ‘open’ and capable of performing photosynthesis (Huñer et al. 1998). As qL decreases, photosynthetic ability decreases due to the reduced number of ‘open’ PSII reaction centers. The redox state of PQ (also termed ‘excitation pressure’) is thought to be an early sensor for imbalances in photostasis and can be estimated by measuring qL.

1.4 PHOTOACCLIMATION

Oxidative stress response mechanisms are distinct at the level of time scale and can be classified into mechanisms for short-term, acute oxidative stress occurring over seconds to minutes or long-term, constitutive stress occurring over hours to years. Short-term response mechanisms are non-heritable adjustments to physiology and biochemistry that focus mainly on avoiding ROS production (Niyogi 2009). In contrast, organisms exposed to long-term stress can induce transcriptional and translational adjustments to many photosynthetic and metabolic pathways to permit long-term acclimation to the particular stress (e.g. induction of ROS degradation pathways) (Niyogi 2009). Photochemical and non-photochemical quenching through enhanced CO$_2$-dependent “photochemical sinks” or heat dissipation are common mechanisms
under either long- or short-term photooxidative stress for maintenance of photostasis; however, these two mechanisms alone may not be enough and alternative electron transport pathways may be induced (Ivanov et al. 2012).

1.4.1 ALTERNATIVE ELECTRON TRANSPORT PATHWAYS

Alternative electron transport pathways further assist in maintaining photostasis and oxidative stress avoidance in photosynthetic organisms by modulating the flow of electrons from classic, linear electron transport to alternative electron transport pathways. When linear electron flow (i.e., from water to NADP⁺) is disrupted due to environmental stress, these alternative electron transport pathways are induced. They include cyclic electron flow (CEF) around PSI, CEF within PSII (plastoquinol terminal oxidase, PTOX), as well as the ascorbate-glutathione pathway (ASH-GSH pathway; also known as the Mehler reaction or the water-water cycle; Makino et al. 2002, Asada 1999, Mehler 1957). Photorespiration can also act as an alternative electron sink for excess photochemical energy, but can further increase the levels of H₂O₂ (Sirikhachornkit and Niyogi 2010, Apel and Hirt 2004).

1.4.1.1 CYCLIC ELECTRON FLOW

Cyclic electron flow (CEF) of electrons around PSI protects against photoinhibition by generating a high ΔpH (low thylakoid pH), which enhances potential for ΔpH-mediated energy dissipation via PSII as well as ATP formation (Niyogi 2009, Heber and Walker 1992, Munekage et al. 2002, Finazzi et al. 2001). CEF generates the pH gradient and reduces ROS production by rerouting electrons from PSI to the PQ pool without reducing NADP⁺ (Joet et al. 2002). It has been observed as an important alternative electron flow pathway in several cyanobacterial and algal species (Ravenel et al. 1994, Joet et al. 2002). Cyclic electron flow has also been observed around PSII as a dissipation of excess energy (Ivanov et al. 2012).

1.4.1.2 PLASTOQUINOL TERMINAL OXIDASE (PTOX)

The plastoquinol terminal oxidase (PTOX) is an alternative electron sink involved in the chlororespiratory pathway and carotenoid biosynthesis (Ivanov et al 2012). PTOX can help reoxidize the PQ pool and induce CEF- like electron flow around PSII. Water split at PSII liberates protons (H⁺) that PTOX uses along with electrons directly transferred from the PQ pool.
to oxygen to regenerate water, thus creating a proton motive force gradient as in CEF (Bailey et al. 2008, Ivanov et al. 2012). PTOX has been observed to help maintain electron flow when CEF around PSI was low in marine algae *Synechococcus* WH8102, green algae *Ostreococcus* and a higher plant *Arabidopsis thaliana* under iron deficiency, high light and low temperature stress, respectively (Bailey et al. 2008, Ivanov et al. 2012, Cardol et al. 2008).

**1.4.1.3 THE ASCORBATE-GLUTATHIONE PATHWAY**

The ascorbate-glutathione pathway, also known at the water-water cycle, is an important ROS defense pathway with dual functions. It not only detoxifies hydrogen peroxide but also reduces redox potential within the photosynthetic electron transport chain by consuming NADPH. Electrons transferred from PSI to oxygen can create a high redox potential (i.e., high NADPH/NADP⁺ ratio) which can induce photoinhibition and ROS formation (Apel and Hirt 2004, Asada 1992). The ascorbate-glutathione pathway can reduce redox potential and generate additional final electron acceptors (NADP⁺). The name water-water cycle comes from the process of reducing H₂O₂ to H₂O by ascorbate peroxidase (APX) at PSI using electrons generated by the splitting of water at PSII (Asada 2000). This pathway is essential for the regeneration of ascorbate, the reducing agent that helps APX reduce harmful hydrogen peroxide to water. When APX reduces hydrogen peroxide to water it oxidizes ascorbate to monodehydroascorbate (MDHA). MDHA can be reduced back to ascorbate by monodehydroascorbate reductase (MDHAR) or via dehydroascorbate reductase and glutathione reductase. In these cases, NADPH is the terminal electron donor that catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) via glutathione reductase (Fig. 1.2). Additionally GSH can act as reducing equivalents for the glutathione peroxidase (GPX) detoxification of H₂O₂ (Apel and Hirt 2004).

**1.4.2 SHORT-TERM STRESS PHOTOACCLIMATION**

Short-term stress response mechanisms in photosynthetic organisms occur rapidly (seconds to minutes) in response to changing environmental factors that perturb energy balance and redox poise. Common short-term stress response mechanisms are photochemical quenching, thermal energy dissipation, state transitions and cyclic electron flow (Niyogi 2009, Dolhi et al. 2012, Morgan-Kiss et al. 2006). The D1 repair cycle is also essential in maintaining photostasis by
constantly replacing PSII D1 protein (psbA) to prevent excessive PSII damage (Pocock et al. 2007).

Thermal dissipation (qE) is a rapid and reversible process involving $\Phi_{NPQ}$ and $\Phi_{NO}$, which dissipate excess energy as heat to prevent ROS formation (Fig. 1.1). These two thermal dissipation mechanisms along with photochemical quenching of the PSII and PSI reaction center antennas play critical roles in energy partitioning and avoidance of ROS production (Morgan-Kiss et al. 2006). $\Phi_{NPQ}$ heat dissipation occurs through trans-thylakoid $\Delta$pH-induced xanthophyll cycle and antenna quenching while $\Phi_{NO}$ dissipates heat through non-inducible xanthophyll cycle-independent processes such as photorespiration, reaction center quenching and the ASH-GSH pathway (Szyszka et al. 2007, Ivanov et al. 2008, Apel and Hirt 2004).

Thermal dissipation along with many other short-term stress response mechanisms rely on maintenance of low thylakoid pH (i.e., high $\Delta$pH). The $\Delta$pH is essential for generation of ATP as well as the activate the xanthophyll cycle and other carotenoids that maintain the energy balance between utilization and heat dissipation of absorbed light (Niyogi 2009). Low thylakoid pH is also required for state transitions (qT), which involve altered phosphorylation of the two photosystems to regulate excitation pressure and maintain photostasis. In State 1 LHCII and PSII are associated while in State 2 LHCII and PSII are dissociated and excitation of PSI is favored (Morgan-Kiss et al. 2002a).

Failure of short-term avoidance mechanisms to reduce stress can cause damage to the PSII reaction center protein D1. Damage to PSII reduces photochemical capabilities inducing photoinhibition, over-reduction of the PQ pool and ROS generation. D1 damage and photoinhibition can slowly be reversed by the D1 repair cycle due to constant turnover and de novo synthesis of new D1 proteins (Pocock et al. 2007, Sirikhachornkit and Niyogi 2010). Low temperature stress has been observed to reduce the rate of D1 repair cycle through inhibition of precursor synthesis (Pocock et al. 2007, Aro et al. 1990). Additional stresses could also influence D1 repair and cause PSII damage.

1.4.3 LONG-TERM STRESS PHOTOACCLIMATION

Long-term stress response mechanisms are induced when photosynthetic organisms are exposed to environmental stress for hours to years; therefore, long-term stress response mechanisms are heavily based on photoacclimation to restore and maintain photostasis. There
are primarily two types of long-term stress response mechanisms: (i) photosynthetic adjustments, and (ii) ROS detoxification.

Photochemical adjustments include alteration of the LHCI/LHCII size, increased CO₂ fixation or alternative electron sinks as well as alterations in energy partitioning. Alterations in the photosystem reaction centers and light harvesting centers can be easily monitored by the Chl a/b ratio. LHCII is associated with Chl b while LHCI is associated mainly with Chl a. Altering the LHC size can help maximize efficiency of photosynthetic electron transport while maintaining low excitation pressure (Szyszka et al. 2007). Under oxidative stress conditions a mesophilic algae *Chlorella vulgaris*, was observed to double its Chl a/b ratio, thereby decreasing its LHCII antenna size (Maxwell et al. 1995a). By decreasing its LHCII antenna size the organism decreased its capacity to absorb higher levels of irradiance, further reducing photoinhibition. Reduction in LHCII absorption capacity is also accomplished by modification of the pigment content of the light harvesting antenna by replacing Chl with other pigments such as carotenoids and carotenes. Some photoautotrophs acclimate to stress by increasing their capacity for CO₂ assimilation or photorespiration by elevating levels of Calvin cycle enzymes, such as RubisCO (Hüner et al. 1998). Additionally, alternative electron transport mechanisms, as explained in section 1.2.1, can also play important roles as long-term electron sinks.

ROS detoxification can occur enzymatically or non-enzymatically. Enzymatic defense mechanisms, catalyzed by antioxidant defense enzymes (AOE), result in detoxification of ROS. They include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), thioredoxin peroxidase (TRX) and catalase (CAT). SOD is an important AOE that converts superoxides into H₂O₂, which can be further converted into H₂O by CAT, GPX, TRX, and APX (Fig. 1.1). These AOE are regulated via gene expression, which are commonly up-regulated during acclimation to long-term stress (Niyogi 2009). Studies have shown that gene expression can be triggered by both the redox state of the PQ pool as well as transthylakoid ΔpH (Maxwell et al. 1995b, Wilson et al. 2000). Non-enzymatic scavengers like carotenoids, ascorbates and thiols also play essential roles in ROS detoxification, such as the ASH-GSH pathway (Singh and Tuteja 2010, Barros et al. 2005).
1.5 STUDY ORGANISMS

The Antarctic green alga *Chlamydomonas raudensis* UWO241 has emerged as a model for cold adaptation of photosynthesis due to its unique adaptive strategies to extreme environments of permanent low temperature and low light (Morgan-Kiss et al. 2006). In addition, it has a closely related mesophilic relative with an identical 18S rRNA sequence, *C. raudensis* SAG49.72 (Pocock et al. 2004). *C. raudensis* UWO241 is an obligate psychrophilic green algae that was isolated from Antarctic Lake Bonney (McMurdo Dry Valleys, Antarctica), a permanently ice covered lake with high salt concentration and extreme low temperatures. Therefore, UWO241 is adapted to permanent low temperatures (0 to 15°C), low light (≤ 20 µmol photon m² s⁻¹) and high salt (700 mM NaCl; Table 1.1) (Morgan-Kiss et al. 2006, Neale and Priscu 1995). Due to its unique adaptation to multiple permanent extreme conditions, UWO241 is not just a psychrophile but rather a polyextremophile. *C. raudensis* SAG49.72 was isolated from a temperate lake in Europe, is non-halotolerant and adapted to moderate light (~120 µmol photon m² s⁻¹) and temperatures (15-30 °C) (Morgan-Kiss et al. 2006, Pocock et al. 2011, Dolhi et al. 2013, Pocock et al. 2007, Szyszka et al. 2007, Possmyer et al. 2011). Studying these two closely related species isolated from diverse environments provides a unique opportunity to compare oxidative stress response mechanisms and further determine adaptations in cold adapted algae.

1.6 STRESS RESPONSE IN C. RAUDENSI S UWO241

UWO241 has been studied over the last 20 years as a model organism for cold adaptation. Photosynthetic organisms such as UWO241 that reside at constant low temperatures are exposed to potentially permanent photoxidative stress due to an energetic imbalance between temperature-independent photochemical reactions and temperature-sensitive enzymatic reactions (Dolhi et al. 2013). Some cold adapted enzymes exhibit high catalytic rates (k_cat), low activation energy requirements (E_a), increased structural flexibility or plasticity and low turnover rate with increased abundance (Dolhi et al. 2013, D’amico et al. 2006, Doyle et al. 2001, Siglioccolo et al. 2010, Possmyer et al. 2011). UWO241 has the potential to support increased enzymes and enzymatic activity due to presence of higher cellular ATP levels and synthetic pathways to maintain ATP requirements (Dolhi et al. 2013, Morgan-Kiss et al. 2002a, Parry and Shain 2011).
Cyclic electron transport is also relatively high in UWO241 providing additional proton gradient for ATP production (Dolhi et al. 2013, Morgan-Kiss et al. 2002b).

Since UWO241 has evolved to persist under a “permanent energy imbalance” for at least a thousand years, it is likely that it possesses mechanisms to deal with these environmental stresses (Neale and Priscu 1995). Thus, UWO241 may contain enhanced or novel adaptive abilities to acclimate to constitutive, long-term stress (i.e., stress acclimation). On the other hand, adaptation to permanent environmental stress may have led to a loss of short-term response mechanisms to variability in environmental stresses. These predictions are supported by earlier studies that reported that UWO241 lacks several well-conserved long and short-term acclimatory mechanisms like alteration of LHCII and state transitions (Pocock et al. 2011, Dolhi et al. 2013, Wilson et al. 2006, Morgan-Kiss et al. 2002a, Ivanov et al. 2012, Davis et al. 2013, Bonente et al. 2008). UWO241 lacks typical long-term acclimation by maintaining a low Chl a/b ratio in conjunction with a large LHCII, as well as constitutively low levels of PSI and PSI-associated light harvesting proteins (Morgan et al. 1998, Szyszka et al. 2007). A large LHCII should favor adaptation to low light conditions; however, a large antenna also increases the potential for ROS production under excessive light conditions (Neale and Priscu 1995). UWO241 does not perform state transitions and is locked in State I. This is likely because UWO241 does not phosphorylate LHCII or other PSII associated polypeptides, but phosphorylates a threonine residue on an unidentified polypeptide, which is likely associated with PSI (Morgan-Kiss et al. 2005, Morgan-Kiss et al. 2002b, Szyszka et al. 2007).

UWO241 has also been observed to contain unique and novel defense mechanisms compared to SAG49.772 acclimated to extreme temperatures, light and salt concentrations. Szyszka et al. (2007) observed the importance of inducible ΦNPQ associated with the xanthophyll cycle and antenna quenching in mesophilic strain SAG49.72, while UWO241 appears to rely more heavily on the less understood non-regulated ΦNO for survival under high temperature acclimation. Pocock et al. (2010) and Possmayer et al. (2011) suggest that UWO241 has increased phenotypic plasticity and survival due to redox sensing and signaling. Finally, recovery from low temperature stress in UWO241 occurs due to a novel D1 repair cycle despite the limited capabilities of the D1 repair cycle in other Chlamydomonas species (Pocock et al. 2007). These results support the opportunity for additional novel stress response mechanisms in extremophilic organisms such as C. raudensis UWO241.
1.7 THESIS OBJECTIVE

The purpose of this study is to expand our understanding of adaptation of the photosynthetic apparatus to permanent environmental stress and to assess the consequences of long-term adaptation to extreme environments on an organism’s ability to avoid short-term photooxidative stress. I hypothesize that as a consequence of adaptation to permanent stress the psychrophilic organism UWO241 has distinct and/or enhanced long-term stress acclimatory mechanisms to avoid oxidative injury while having limited ability to acclimate to short-term photooxidative stress compared to its close mesophilic relative, SAG.49.72.

Two aims will be addressed:

Aim 1. Characterization of long-term stress acclimation in psychrophilic UWO241 and mesophilic SAG49.72 *Chlamydomonas raudensis* strains.

Figure 1.1. A simplified overview of the impact of oxidative stress on the photosynthetic apparatus. (A). Under control conditions (ie. non-stress growth condition), linear electron flow (LEF) from PSII to PSI dominates (black arrows), shuttling photosynthetic electrons through metabolic sinks, such as carbon fixation and photorespiration (black boxes). (B). Environmental stressors, such as high light and low temperature, can disrupt LEF and regeneration of NADPH, causing the PQ pool to become over-reduced (shown in yellow), as well as increase excitation pressure and formation of ROS (in red). Photochemical (reaction center quenching, state transitions; redistribution LHCII) and non-photochemical (heat dissipation $\Phi_{NPQ}$; dark red arrow) quenching help reduce excitation pressure and ROS formation. ROS defense mechanisms (green boxes) are both enzymatic (SOD, GPX, APX, TRX, CAT) and non-enzymatic (carotenoids). Alternative electron sink pathways, such as cyclic electron flow (CEF), ascorbate-glutathione pathway (ASH GSH pathway) and plastoquinol terminal oxidase (PTOX; dotted lines) can also be induced to reduce oxidative stress.
Figure 1.2. Ascorbate glutathione cycle (taken from Locato et al. 2013). Hydrogen peroxide is scavenged by ascorbate and enzyme ascorbate peroxidase (APX). Ascorbate is then regenerated via enzymes monodehydroascorbate reductase (MDHAR) or dihydroascorbate reductase (DHAR) and glutathione reductase (GR). NADPH is the terminal electron acceptor.
Table 1.1. List of multiple permanent environmental stresses faced by *C. raudensis* UWO241 in its natural habitat and its adaptations to permanent stress. Putative genes were identified from a new cDNA sequence library generated in UWO241. Numbers in parentheses indicates # of unique homologues for a given gene identified in a BlastX search. (HSP, heat stress proteins; IBP, ice binding protein; GPX, glutathione peroxidase; GR, glutathione reductase; APX, ascorbate peroxidase; AOX, alternative plant oxidase; CAT, catalase; TR, Thioredoxin Reductases).

<table>
<thead>
<tr>
<th>Extreme Condition</th>
<th>Growth Limitations</th>
<th>Physiological Consequences</th>
<th>Putative identified genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Temperature</td>
<td>Min = 1 °C; Opt = 10 °C; Max=16 °C</td>
<td>Psychrophilic; high levels of polyunsaturated fatty acids; thermolabile photosynthetic apparatus</td>
<td>HSPs (19); IBPs (3); antifreeze protein (1); fatty acid desaturase (18);</td>
</tr>
<tr>
<td>High Salinity (NaCl)</td>
<td>Min=10 mM Opt=100 mM Max=1300 mM</td>
<td>Halotolerant; high ATP production; high PSI-cyclic electron transport; PSI-P</td>
<td>salt tolerance proteins (3); Na⁺ exchange (7)</td>
</tr>
<tr>
<td>Light Quality</td>
<td>Blue light requirement</td>
<td>High PS II:PS I ratio; reduced PS I and LHCI; inability to grow in red light</td>
<td>LHCa (11); blue light photoreceptors (3)</td>
</tr>
<tr>
<td>High dissolved Oxygen</td>
<td>None known</td>
<td>Unknown</td>
<td>GPX (5) , GR (4) APX (2), AOX (2), CAT (2); thioredoxins (40); TR (7) peroxiredoxin (7)</td>
</tr>
<tr>
<td>Irradiance</td>
<td>Min: 1 µmol m⁻² s⁻¹ Opt: 20 µmol m⁻² s⁻¹ Max: 250 µmol m⁻² s⁻¹</td>
<td>Large light harvesting antenna; efficient energy transfer; limited qE energy quenching; reduced eyespot</td>
<td>LHCb (15)</td>
</tr>
</tbody>
</table>
CHAPTER TWO

CHARACTERIZATION OF LONG-TERM STRESS RESPONSE MECHANISMS IN THE PSYCHROPHILE CHLAMYDOMONAS RAUDENSIS UWO241 IN COMPARISON TO THE MESOPHILE CHLAMYDOMONAS RAUDENSIS SAG49.72
2.1 INTRODUCTION

Extremophiles, such as *C. raudensis* UWO241, have typically evolved to survive multiple constitutive environmental stressors. Constitutive or long-term stress can be defined as an environmental stress, which an organism is exposed to for long periods of time (hours to years). Regardless of the type of stress, a common outcome of exposure to an environmental stress in a photosynthetic organism is the disruption of photostasis, which can lead to formation of reactive oxygen species (ROS). Long-term stress acclimation mechanisms in algae have been thoroughly described in the model organism *C. reinhardtii*, and involve alterations in photosynthetic capacity and cellular metabolism to limit ROS production as well as detoxification of ROS (Niyogi 2009). Acclimation mechanisms include modulations in photosynthetic gene expression that reduce the light harvesting complex (LHC) capacity, altered PSII:PSI ratios, and shifts in pigmentation that favor energy dissipation over absorption. In combination with down-regulation of photosynthesis, cells may also upregulate ROS detoxification process, including activity of several antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), thioredoxin reductase (TRX), glutathione peroxidase (GPX) and ascorbate peroxidase (APX) to increase the capacity of the cell to detoxify ROS (Davis et al. 2013, Pocock et al. 2011, Pocock et al. 2004, Niyogi 2009).

The Antarctic green alga, *C. raudensis* UWO241, is exposed to a number of long-term environmental stress conditions in its natural habitat, including low temperatures, high salinity, nutrient deficiency, extreme shade and high oxygen (Morgan-Kiss et al. 2006). Whereas past studies have thoroughly described physiological adaptation to this unusual environment (reviewed in Dolhi et al. 2013), there have been few studies that directly tested the impact of long-term stress acclimation in UWO241. Compared with the mesophilic sister strain, SAG49.72, UWO241 exhibits differential responses to long-term stress, including different energy partitioning strategies, photosystem stoichiometry and polypeptide phosphorylation to maintain photostasis (Szyszka et al. 2007; Pocock et al. 2011).

This chapter will extend previous work on UWO241 by comparing long-term acclimatory responses in UWO241 vs. SAG49.72 to three environmental stresses that induce photooxidative stress by disrupting energy balance via manipulation of salinity, low temperature, and high light. Experiments will monitor: (i) the capability of each organism to rebalance
photostasis and thus avoid ROS production, and (ii) whether ROS detoxification pathways are induced under environmental stress.

2.2 METHODS

2.2.1 Long-term stress growth conditions. Cultures of the psychrophilic *C. raudensis* UWO241 (P) and the mesophilic *C. raudensis* SAG49.72 (M) algal strains were grown under four conditions, representing one control and three long-term stress conditions. The long-term stress conditions were chosen for each organism to reflect the maximum level of a particular stress that the organism could tolerate and still exhibit exponential growth and high photochemical activity, indicating full acclimation. The long-term stress conditions were: (i) high light [HL: 250 μmol m$^{-2}$ s$^{-1}$ (P), 500 μmol m$^{-2}$ s$^{-1}$ (M)], (ii) low temperature [LT: 2 °C (P), 11 °C (M)], (iii) high salt [HS: 700 mM NaCl (P), 100 mM NaCl (M)] (Fig. 2.1). UWO241 and SAG49.72 were grown under respective control conditions, 8 °C, 50 μmol m$^{-2}$ s$^{-1}$ (P) and 20 °C, 50 μmol m$^{-2}$ s$^{-1}$ (M), and reinoculated into fresh media before being transferred to stress conditions. Cultures were grown in 250 mL Pyrex tubes suspended in a temperature-regulated aquarium under continuous CO$_2$ in Bold’s Basal Medium (BBM) (Nicholas and Bold 1965, Morgan et al. 1998). Growth rate was monitored as change in optical density at 750 nm. Daily growth and photochemical measurements were taken. All other measurements were performed on mid-log phase cultures.

2.2.2 Chlorophyll (Chl) analysis. Samples (2 mL) were collected from cultures during mid-log phase of growth, centrifuged at 16,000 x g for 5 min at room temperature and stored at -80°C. Pellets were resuspended in 1 mL 90% (vol/vol) acetone on ice and homogenized using a bead beater for 2 cycles of 45 s, chilled in a ice bath between cycles to minimize thermal stress. Acetone extracts were then centrifuged at 16,000 x g for 2 mins and the supernatant was transferred to a fresh tube. The supernatant was used for Chl a and Chl b determination using methods from Jeffery & Humphrey (1975). Pellets were saved for total protein concentration determination.

2.2.3 Protein concentration determination. Pellets from Chl extractions were resuspended in 0.25 M NaOH, incubated at room temperature for 10 mins, then neutralized with an equal
volume of 0.26 M HCl 200 μL of sample was added to 600 μL of 20 mM potassium phosphate buffer (pH 7.0) and 200 μL Bradford dye and incubated for 15 min. Protein concentrations were determined spectrophotometrically at a wavelength of 595 nm and converted to mg/mL using a standard curve of bovine serum albumin (BSA) protein standard (Bradford 1976).

2.2.4. Chlorophyll fluorescence. Steady state Chl-a fluorescence was measured using a pulse amplitude modulated Chl a fluorescence detection system (Dual PAM-101, Chlorophyll Fluorescence & P700 Photosynthesis Analyzer, Walz, Germany). For PSII analysis, 2 mL of algal culture was collected and dark-adapted in a water-jacketed cuvette in the presence of 4 mM NaHCO3 for 2 min. For PSI analysis, 15 mL of algal culture was dark-adapted for 5 min in a circulating water bath and filtered onto a Whatman GF/C 25mm filter (Cat No 1822-025). PSI activity was measured as the change in absorbance at 820 nm (Morgan-Kiss et al. 2002b). Energy partition in PSI was determined though ΦPSI, ΦNA, ΦND (ΦPSI + ΦNA + ΦND=1). Quantum yield of PSI electron transport is ΦPSI. Electron transport limited by donor side between PSII and PSI limitation was determined by ΦND and acceptor side limitation at PSI was determined by ΦNA (Schreiber and Klughammer 2008). Other parameters (Fv/FM, qL, ΦPSII, ΦNPQ, ΦNO) were calculated using the saturating pulse Chl a fluorescence method from standard induction and light curves (Baker 2008). Fv/FM measures maximum quantum efficiency of PSII photochemistry, while qL is the coefficient of photochemical fluorescence quenching and is an estimate of the proportion of PSII centers that are oxidized. Energy partitioning within PSII reaction centers was determined by monitoring three parameters, ΦPSII, ΦNPQ, ΦNO (ΦPSII + ΦNPQ + ΦNO= 1). ΦPSII is the quantum yield of PSII electron transport, while ΦNPQ and ΦNO are quantum yields of energy loss through regulated non-photochemical quenching and non-regulated photochemical quenching, respectively (Schreiber and Klughammer 2008, Szyzska et al. 2007).

The quantum yield of cyclic electron flow (CEF) was calculated from the difference between quantum yield of PSI and PSII; ΦCEF = ΦPSI - ΦPSII (Wang et al. 2013). The relationship between CEF and linear electron flow (LEF) was determined by change in ratio of ΦCEF)/ ΦPSII.
2.2.5. Low temperature Chl a fluorescence (77K). Algal cultures (~250 µL) from mid-log phase cultures were transferred to NMR tubes, dark adapted for 10 mins and flash frozen in liquid nitrogen. Low temperature fluorescence spectra were obtained at the excitation wavelength of 435 nm for Chl a fluorescence detection using a Perkin Elmer Luminescence Spectrometer (LS50B) (Buckinghamshire, England) equipped with liquid nitrogen accessory (Morgan, et al. 1998, Morgan-Kiss et al. 2002a). Decompositional analysis of fluorescence emission spectra in terms of five Gaussian bands was performed by a non-linear least squares algorithm according to Morgan-Kiss et al. (2002a) using the program OriginPro 8.5.1.

2.2.6. SDS-PAGE and immunoblotting. For electrophoretic separation of proteins, 25 mL of culture was harvested from mid-log phase cultures by centrifugation. Total protein was extracted by addition of 1.5 mL of 1X LDS extraction buffer containing 140 mM Tris base, 105 mM Tris–HCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 2% lithium dodecyl sulfate (LDS), 10% glycerol and 0.1 mg/mL PefaBloc SC (AEBSF) protease inhibitor (Roche). Samples were bead beater disrupted for 5 x 30 sec with ice incubation between each cycle to prevent sample heating. The lysates were centrifuged for 1 min at 1,1912 x g at 4°C to remove insoluble debris. Protein concentration was determined via DC compatible Bradford assay (Bio-Rad) with 10 mg/mL BSA standards (Lowry et al. 1951). Samples were loaded on an equal protein basis (5 µg total protein per lane) and separated on a 15% (w/v) SDS-PAGE polyacrylamide resolving gel and 8% (w/v) polyacrylamide stacking gel using a Mini-Protein apparatus (Bio-Rad, Laemmli 1970).

Gels were transferred to methanol-activated PVDF membrane and blocked overnight in a solution of Tris-Buffered-Saline (TBS, 0.2 M Tris-HCl; 1.5 M NaCl; pH 7.5) with 0.05% Tween 20 and 5% non-fat milk. PVDF membranes were probed with 1° antibodies raised against PsbA (1:10,000 dilution), RubisCO (RbcL) (1:10,000) or ascorbate peroxidase (APX) (1:10,000) (Agrisers Vannas, Sweden) for 1 hour vigorously shaking at room temperature. After primary antibody incubation, PVDF membranes were washed with TBS: Tween solution for 15 minutes (X2) and 5 minutes (X2). Membranes were then exposed to anti-rabbit 2° antibody Protein A conjugated to horseradish peroxidase (HRP) (1:10,000 dilution) (Sigma-Aldrich) for 1 hr followed by a similar set of TBS:Tween washes. To visualize the targeted proteins, PVDF membranes were incubated with ECL Select™ Western Blotting Detection Reagent (Amersham, Cat No. RPN2235) for 5 minutes and detected on X-Ray film. Exposed films were developed,
scanned and protein abundance was semiquantitatively analyzed for densitometry using ImageJ (http://imagej.nih.gov/ij/) as previously described (Morgan et al. 1998).

2.2.7. Glutathione Reductase Assay. GR activity was measured using a glutathione reductase assay kit following manufacture’s instructions (Cayman Chemicals, Ann Arbor). Ten milliliters of mid-log phase culture was collected and centrifuged at 2,000 x g for 5 min at 4 °C, flash-frozen and stored at -80 °C. Cell pellets were resuspended in 1.5 mL of GR assay buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.5) and bead-beater disrupted 4 x 30 sec with ice breaks between. Twenty microliters of supernatant was mixed with 100 μL GR assay buffer and 20 μL oxidized glutathione. Positive assay controls contained 20 μL glutathione reductase enzyme, and non-enzymatic control contained only assay buffer. The reactions were initiated with 50 μL NADPH, and absorbance was read every minute for 10 min at 340 nm (25°C). Activity was calculated by Δ A340 min⁻¹mg⁻¹ protein using an NADPH extinction coefficient of 0.00373 μM⁻¹. Total protein was determined using Bradford assay from cold acetone extraction as described in 2.2.3.

2.2.8. Ascorbate peroxidase activity. APX activity was measured according to Venisse et al. (2001) with some modifications. Sample extracts were prepared as described for the GR activity assay. Ten microliters of supernatant was added to 190 μL of reaction buffer (50 mM potassium phosphate buffer, pH 7.8), supplemented with 0.5 mM ascorbic acid and 0.1 mM H₂O₂. Oxidation of ascorbate was monitored spectrophotometrically as a decrease in absorbance at 290 nm (extinction coefficient 0.00168 μM⁻¹) over 10 min to determine APX activity (Venisse et al. 2001).

2.2.9. Quantitative PCR of glycerol synthase (serB). Expression of serB, glycerol synthase, was quantified by real time quantitative PCR in cultures of UWO241 grown under variable salinity. Cultures were grown at 8 °C and 50 μmol m⁻² s⁻¹ in BBM supplemented with a range of salt concentrations (10 mM, 300 mM, 700 mM and 1300 mM NaCl) until samples reached mid log. Total RNA was extracted using Qiagen RNeasy mini kit (No. 74104) as per manufacturer’s instructions. Residual genomic DNA was removed by Ambion DNase. RNA was reverse transcribed to cDNA by iScript cDNA synthesis kit (Bio-Rad, CA) as specified by manufacturer.
Several reference genes were also quantified in order to normalize serB transcript abundance across samples using the ΔΔCq method (Haires and Kelley 2010, Liu et al. 2012; Table 2.4). Reference genes used were histh2b (histone H2B), rps10 (40s ribosomal protein S10), rpl19 (60s ribosomal protein L19) and gap3 (glycerol 3-phosphate dehydrogenase). The ΔΔCq method normalizes data across treatments by subtracting expression of reference genes whose expression should remain constant across treatments.

2.2.10. Lipid analysis. Oil storage bodies were visualized in UWO241 and SAG49.72 cultures grown under control vs. long-term stress conditions using BODIPY 505/515 lipophilic fluorescent dye (4,4-difluro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, Invitrogen Molecular Probes, Carlsbad). Two μL of the 1 mM solution of BODIPY 505/515 dissolved in anhydrous dimethyl sulfoxide was added to a dark tube containing 1 mL of mid-log algal culture. Stained samples were examined and imaged using a Zeiss Laser Scanning Microscopy (LSM 710) system. A 488 nm blue laser excited the samples; BODIPY fluorescence was visualized using an emission of 510 to 530 nm, while Chl a fluorescence was detected using emission wavelength no longer than 650 nm (Copper et al. 2010, Brennan 2012 Govender et al. 2012, MacIsaac et al. 1993). Lipid body counts per cell and diameters were quantified through densitometry using ImageJ.

2.2.11. Statistical Analyses. Means and standard deviations (S.D.) were calculated and presented for each treatment with three biological replicates. The statistical significance was determined using Student’s paired t-test (OriginPro 8.5.1) between stress conditions and control within a single organism as well as between stress conditions in both organisms. Statistical significance was accepted when P value was less than 0.05.
2.3 RESULTS

2.3.1 Growth physiology under long-term stress. To compare the mechanisms by which the psychrophilic UWO241 and the mesophilic SAG49.72 acclimate to long-term environmental stress, the two strains were grown under control growth conditions and then shifted to one of three different stress conditions, representing high light (HL), low temperature (LT) and high salt (HS; Fig. 2.1). First, it was confirmed that both strains could fully acclimate to each stress conditions by comparing photochemical performance and growth rates in cultures acclimated to control vs. high light, high salinity or low temperatures. UWO241 and SAG49.72 successful acclimated to all long-term stress conditions as reflected in their ability to exhibit exponential growth and achieve high PSII photochemical efficiency (Fv/Fm) under stress conditions. However, it should be noted that UWO241 exhibited significantly lower Fv/Fm values compared with SAG49.72 under all growth conditions (Table 2.1). Despite the fact that UWO241 was grown under significantly lower temperature compared with SAG49.72, the two strains generally exhibited comparable growth rates under control and stress condition, with the exception of high light treatment in SAG49.72 which exhibited a 3-fold increase in growth rate (Fig. 2.2). Relative to control cultures, low temperature treatments induced a significant decrease in photochemical ability (Fv/Fm), total Chl (μg/mL), and total protein (μg/mL) in both organisms, while high light induced a significant reduction in these parameters in UWO241 only (Table 2.1). Ratios of Chl a/b reflect the relative size of the LHCII complexes, since Chl b is exclusively located in the light harvesting apparatus (Dolhi et al. 2013). Chl a/b ratios were constitutively lower in UWO241 vs. SAG49.72 under all growth conditions (Fig. 2.3). In addition, SAG49.72 cultures grown under all long-term stress conditions exhibited higher Chl a/b ratios compared with control cultures; although, this difference was only significant between the control and low temperature-grown cultures (Fig. 2.3).

2.3.2 Photosynthetic protein abundance. PsbA and RubisCO were chosen for semiquantitation via immunoblotting as indicators of photosynthetic capacity at the level of the photochemistry vs. carbon fixation, respectively. In general, changes in abundance of either photosynthetic protein were statistically insignificant. SAG49.72 exhibited an increase in RbcL under long-term
high light treatment but a decrease in RbcL under low temperature treatments relative to control-grown cultures (Fig. 2.4).

2.3.3. Photosystem II function and energy partitioning. Analyses of the photochemical performance in cultures acclimated to long-term stress conditions revealed some similarities as well as some differences between the organisms. UWO241 had statistically significant lower qL in control, low temperature and high salt conditions compared with SAG49.72, indicative of a lower proportion of “open” or oxidized PSII centers, as well as a constitutively lower $\Phi_{PSII}$ across all treatments. On the other hand, qL was comparable in control and stress conditions in UWO241, indicating that the psychrophile maintains a relatively oxidized PQ pool when grown under high light, lower temperature or high salinity (Fig. 2.5A). In contrast, the high light condition resulted in a significant reduction of qL in SAG49.72 (Fig. 2.5B). This decrease in the oxidation state of the PQ pool was accompanied by a reduction of $\Phi_{PSII}$ in favor of increased non-regulated $\Phi_{NO}$ in SAG49.72 and regulated $\Phi_{NPQ}$ in UWO241 (Fig. 2.5C-2.5D). Low temperature and high salinity treatments in both species exhibited comparable energy partitioning relative to control cultures (Fig. 2.5C-2.5D). Thus, both organisms were most responsive to the high light conditions at the level of adjustments in energy partitioning.

2.3.4. Photosystem I functioning and energy partitioning. The Dual-PAM-100 fluorometer allowed for simultaneous monitoring of changes in both Chl fluorescence (e.g., PSII measurements) and P700 absorbance (e.g., PSI measurements). Specifically, three quantum yields can be measured for PSI: $\Phi_{PSI}$, PSI photochemical quantum yield; $\Phi_{ND}$, quantum yield of non-photochemical energy dissipation in PSI due to donor side limitation; and $\Phi_{NA}$, quantum yield of non-photochemical energy dissipation in PSI due to acceptor side limitation. Based on PSI induction curves, the psychrophile, UWO241, consistently exhibited a lower $\Delta A_{820 \text{ nm}}$ compared to the mesophile, SAG49.72 (Fig. 2.6). Both high light and low temperature caused a decline in $\Delta A_{820 \text{ nm}}$ in both algae, while high salinity-grown cultures of SAG49.72 were comparable to control cultures (Table 2.2; Fig. 2.6).

Photochemical analysis of energy partitioning in PSI revealed major differences between the psychrophile and mesophile at the levels of donor vs. acceptor side-dependent down-regulation of PSI (Fig. 2.7). UWO241 cultures grown under control conditions exhibited a
relatively large acceptor side limitation accompanied by a reduced PSI-photochemical quantum yield compared to SAG49.72, suggesting that reoxidation of PSI reaction centers limits PSI activity in UWO241 even under control conditions (Fig. 2.7). In contrast, under high light and to a lesser extent, high salt treatments in both species induced loss in Φ PSI due to a rise in donor side limitation. This effect was exacerbated in high light-grown SAG49.72 cultures. The effect of low temperatures on PSI was different, UWO241 cells exhibited a relatively high Φ PSI, while acceptor side limitation in SAG49.72 reduced Φ PSI (Fig. 2.7).

2.3.5. Effects of long-term stress on energy distribution between PSI and PSII. Low temperature (77K) Chl a fluorescence emission spectra were measured to monitor the energy distribution between major photosynthetic pigment complexes. Figure 2.8, a decomposition analysis of 77K fluorescence mission spectra, shows the best fit of five major spectral components (Table 2.3), which were light harvesting of PSII (1 λ_MAX = 683-685 nm), proximal antenna and core complex of PSII (2 λ_MAX = 695-696 nm; 3 λ_MAX = 697 - 704 nm), PSI core complex (4 λ_MAX = 710-715 nm) and small vibrational transitions (5 λ_MAX = nm F_vib 725 - 740 nm) (Morgan-Kiss et al. 2002a). Peak areas were used to estimate the relative energy coupling between LHCII and PSII (LCHII/PSII core = 1 λ_MAX /[2 λ_MAX + 3 λ_MAX]) and the relative energy distribution between PSII and PSI (PSII/PSI = ([1 λ_MAX + 2 λ_MAX + 3 λ_MAX]/ 4 λ_MAX). All UWO241 cultures exhibited lower LHCII/PSII ratios as well as higher PSII/PSI ratios compared with SAG49.72 (Fig. 2.8A, C, E, G; Table 2.4). Under all growth conditions UWO241 lacked a well-defined PSI. In marked contrast, under all stress treatments the mesophile SAG49.72 exhibited a rise in LHCII/PSII as well as a decrease in PSII/PSI (Fig. 2.8B, D, F, H, Table 2.3), reflecting both a loss in energy transfer efficiency between light harvesting antenna and PSII core reaction centers as well as a redistribution of energy from PSII to PSI.

2.3.6. Cyclic vs. linear electron flow. Photosynthetic organisms possess alternative electron transport pathways in addition to the classic linear electron transport pathway between PSII and PSI. One prevalent alternative pathway is the presence of cyclic electron flow around PSI, which cycles electrons around PSI preventing formation of superoxide and generating a transthylakoid pH that can be used to generate ATP. Our data indicate that cyclic electron flow is higher in UWO241 than in SAG49.72 (Figure 2.9). UWO241 acclimated to high light and low temperature
experienced a 2.1 and 4.1-fold increase in $\Phi_{CEF}/\Phi_{PSII}$ relative to control. UWO241 acclimated to high light significantly upregulated CEF compared to control and SAG49.72 high light conditions (Fig. 2.9). Meanwhile, SAG49.72 reduces its $\Phi_{CEF}/\Phi_{PSII}$ ratio in high salt and significantly in low temperature conditions relative to control suggesting that it relies more heavily on LEF (Fig. 2.9). SAG49.72 high light condition induced a very small increase in CEF/LEF ratio relative to control.

2.3.7. ROS detoxification methods. APX and GR play major roles in ROS detoxification, reducing $H_2O_2$ to $H_2O$ through the ascorbate-glutathione pathway. Immunoblotting indicated that APX protein abundance increased in both organisms under long-term stress acclimation with a significant increase when acclimated to high salt (Fig. 2.10). Immunoblotting also revealed a difference in APX isoenzymes and apparent mass. The blot suggested that UWO241 had two isoforms of APX, which were 25 and 23 kDa, while SAG49.72 contained one APX protein that was 30 kDa (Takeda et al. 2000; Fig. 2.11).

Colorimetric activity assays reveal that APX and GR activities were constitutively increased in the psychrophile UWO241 (Fig. 2.12-2.13). GR activity was higher in high salinity-grown cultures while APX activity was highest in low temperature-grown cultures of UWO241 relative to the control. In contrast, SAG49.72 exhibited no significant change in GR or APX activity in control and stress conditions.

2.3.8. Glycerol Synthase. Photosynthetic organisms that are adapted to survive in high salinity environments often produce high levels of compatible solutes. Production of the osmolyte glycerol as well as quantification of expression levels of a gene encoding a major enzyme in the glycerol synthesis cycle, serB, was examined in cultures of UWO241 grown under a range of salinity concentrations. In addition, expression levels of several reference genes encoding histone H2B, GAPDH, RPS10, and RPL19 were tested for the purposes of normalizing functional gene levels across treatments. Pairwise variation analysis performed by Liu et al. (2012) using the software geNorm suggested two reference genes, containing low cycle threshold (Ct) and high stability values, to be sufficient for qRT-PCR data normalization. Histone H2B, GAPDH and RPS10 were the most stable reference genes with low Ct values of 15.3, 17.2, and 15.8, respectively. Use of two or three reference genes (Histone and RPS10 or Histone, RPS10 and
GAPDH) provided better normalization than a single reference gene (Fig. 2.14A). Levels of serB mRNA and glycerol production exhibited a positive correlation with increase salinity in the growth medium (Fig. 2.14B, 2.14C). In contrast, glycerol production was below detectable levels in cultures of SAG49.72 and there has been no analysis of serB (data not shown). This indicates that the production of glycerol is unique to the psychrophilic alga UWO241 and is an adaptation to its natural environment of high salinity levels.

2.3.9. Lipid production. Photosynthetic organisms store energy as a variety of different compounds. For example, starch is a common storage compound found in chloroplasts. On the other hand, neutral lipids in the form of triacyl glycerols are stored as oil bodies and typically only accumulate in algae when cultures are exposed to extreme nutrient stress (Wang et al 2009). Under control conditions, UWO241 had constitutively higher presence of larger lipid bodies/cell than SAG49.72 with 12.9 ± 3.81 vs. 7.4 ± 3.50 lipid bodies/cell and 2.23 ± 1.20 vs. 0.76 ± 1.07 total lipid volume/cell, respectively (Table 2.5). Acclimation to long-term stress treatments led to alterations in lipid body content in both algal strains. UWO241 acclimated to low temperature conditions exhibited the highest lipid content per cell (Table 2.5). These lipid bodies were not just more abundant but were also large in size having an average radius of 0.36 ± 0.099 µm and lipid body volume of 0.29 ± 0.340 µm³. SAG49.72 acclimated to high light conditions contained a higher total number of lipid bodies/cell (26.9 ± 14.96) than other treatments, however, the average size of the lipid bodies were relatively small, with an average radius of 0.17 ± 0.093 µm and average volume of 0.04 ± 0.023 µm³. In summary, UWO241 control and low temperature treatments contained a larger total number and volume of lipid bodies present per cell than SAG49.72 (Fig. 2.15, Table 2.5).
2.4 DISCUSSION

This study showed that both the psychrophilic UWO241 and mesophilic SAG49.72 exhibited the ability to acclimate to varying long-term stress conditions. However, the level of stress as well as the specific mechanisms employed to allow full acclimation to long-term stress differed between the two organisms. These mechanisms can be divided into two major types (i) photosynthetic modifications to avoid production of excess ROS, and (ii) ROS detoxification.

Remarkably, UWO241 and SAG49.72 exhibited similar growth rates under almost all tested long-term stress conditions, with the exception of high light treatment in SAG49.72, which exhibited significantly higher growth rates (Fig. 2.2). This reflects the unique ability of UWO241 to grow and survive comparably to a mesophilic organism even under more extreme low temperature and high salinity environments. Reduced growth rate under high light in UWO241 was likely due to its permanent adaptation to shade. Permanent shade adaptation may also contribute to the constitutively lower Chl a/b ratio in UWO241. Chl b is primarily associated with LHCII thus UWO241 maintains a constitutively larger, non-adjustable LHCII (Hüner et al. 1998, Dolhi et al. 2012). A larger LHCII would allow more light to enter PSII, which is ideal under low light conditions; however, being unable to adjust the LHCII size can lead to reduced photochemical capability ($F_v/F_m$) under stress. On the other hand, a decrease in the total number of PSII reaction centers (PsbA) in SAG49.72 under low-temperature correlates with the decrease in Chl a/b ratio (LHCII size) (Fig. 2.3, Fig. 2.4), supporting photosynthetic modifications as important mechanisms for reducing photoinhibition in SAG49.72.

Adjustments to the energy distribution between PSII and PSI are essential for acclimation to long-term stress in SAG49.72 but not UWO241 (Fig. 2.8). Previous experiments demonstrated that UWO241 is locked in state 1 and cannot perform state transitions that redistribute energy between photosystems; however, experiments were conducted only under natural high salt conditions (Morgan-Kiss et al. 2002a). UWO241 grown under low salt control conditions were able to induce very small pseudo-state transitions with a passive “spillover” of energy from PSII to PSI (Fig. 2.8; Takizawa et al. 2009). Other stress conditions, low temperature and high light, also failed to result in energy distribution between photosystems regardless of salt concentration present; thus, UWO241 remains a natural variant lacking state transitions and maintaining higher PSII content than PSI regardless of the treatment (Table 2.3). Redistribution of energy in favor of
PSI is sensed when the PQ pool becomes over-reduced; therefore, it is likely that SAG49.72 relieves high excitation pressure by redistribution of energy to PSI. Lack of energy redistribution in UWO241 indicates that this organism does not rely on this major mechanism to relieve high excitation pressure; however, lumen acidification and alternative pathways may be active in UWO241 during stress acclimation (Takizawa et al. 2009).

In contrast with the differential responses at the level of PSII/PSI energy redistribution and modulations in Chl a/b ratios, UWO241 and SAG49.72 acclimated to long-term low temperature and high salt stress maintained similar energy partitioning relative to control conditions. Both strains induced non-photochemical dissipation under high light growth conditions, which could be linked to increased donor side limitation (Fig. 2.5, Fig. 2.7). Our results correspond with a previous study by Szyszka et al. (2007) that showed that high light treatments had the greatest impact on photochemistry by increasing excitation pressure (1-qL) and decreasing ΦPSII; however, in our work, the energy partitioning mechanisms exhibited by UWO241 and SAG49.72 differed with respect to the stress condition. In contrast to Szyszka et al. (2007) we observed UWO241 to increase ΦNPQ, whereas SAG49.72 induced both ΦNPQ and ΦNO. Additionally, UWO241 had constitutively high levels of ΦNO under all growth conditions, which may play an essential role in regulation of permanent excitation energy under low thylakoid ΔpH. Ultimately, UWO241 and SAG49.72 under high light stress rely on both ΦNPQ and ΦNO energy dissipation to regulate excitation pressure and maintain photostasis.

ΦNPQ involves inducible non-photochemical quenching processes such as the xanthophyll cycle, while ΦNO induces non-regulated mechanisms such as reaction center quenching, photorespiration and the ascorbate-glutathione pathway (Ivanov et al. 2008).

A common acclimation mechanism in higher-level plants that has not been extensively studied in algae involves increasing carbon fixation. For example, cold acclimation in cereal grains maintained high photochemical capacity due to increased electron flux through Calvin cycle and carbon fixation (Hunter et al. 1993, Ivanov et al. 2012, Hüner et al. 1998). Our results in algae suggest that SAG49.72 can increase carbon fixation under high light stress acclimation due to the increased abundance of the major carbon fixation protein (RubisCO) along with a 3-fold increase in growth rate (Fig. 2.4, Fig. 2.2), whereas UWO241 did not adjust RubisCO protein levels under any stress conditions.
Since UWO241 did not adjust RbcL levels but still maintained high photochemical capabilities (Fig. 2.2, Table 2.1), we hypothesize the presence of alternative electron sinks downstream of PSII prior to CO₂ fixation. The availability of alternative electron sinks are supported in UWO241 due to its constitutively reduced presence and activity of PSI and high levels of ΦNO energy dissipation under all conditions (Table 2.2, Fig. 2.6, Table 2.3, Fig. 2.5). These alternative electron transport pathways include CEF around PSI, CEF within PSII (e.g. plastoquinol terminal oxidase, PTOX), as well as the ascorbate-glutathione pathway (ASH-GSH pathway) (Makino et al. 2002, Asada 1999, Mehler 1957). These pathways are essential in generating a pH gradient across the thylakoid membrane when limited electron transport acceptors are available at PSI to induce ΦNPQ and ΦNO (Makino et al. 2002, Bailey et al. 2008). Presence of alternative electron transport pathways and their location can be determined via donor and acceptor side limitations measured with the Dual PAM fluorometer. Both UWO241 and SAG49.72 exhibited increased donor side limitation under high light and high salt conditions suggesting the potential presence of an alternative transport pathway(s) between PSII and PSI. PTOX is an alternative electron sink that induces pseudo-CEF around PSII to assist in plastoquinol oxidation. It plays an important role in plant adaptation to high salt, extreme temperatures and high light conditions (Heyno et al. 2009). Two putative PTOX genes have been identified in the UWO241 transcriptome; however, additional research is needed to confirm presence of PTOX like enzymes and activity in both C. raudensis strains.

It has been speculated that LEF is relatively low and CEF is increased in UWO241 (Morgan-Kiss et al. 2006). In this current study the disproportionate ratio of PSII/PSI under all growth conditions suggests that low LEF rates are likely a permanent phenomenon in UWO241 (Table 2.3). Furthermore, based on direct PSI activity measurements, it was estimated that CEF was 1.5-2 fold faster in UWO241 than in C. reinhardtii (Morgan-Kiss et al. 2002a). Our measurements of CEF verify the estimates that CEF is higher in psychrophilic UWO241 compared to mesophilic SAG49.72 acclimated to all stress conditions. CEF appears to be especially important in UWO241 under low temperatures and high light stress (Fig. 2.9) and may help maintain higher PSI quantum yield due to a reduction in acceptor side limitations (Fig. 2.7). On the other hand, reduced Y(CEF)/Y(II) ratio and increased acceptor side limitation (overloaded of PSI) suggests that SAG49.72 is especially limited by reduced CEF at low temperature (Fig. 2.9, Fig. 2.7).
If an organism cannot avoid or reduce ROS production though photochemical modifications then ROS detoxification methods are necessary to reduce ROS and avoid photooxidative damage. This study provides evidence that the psychrophilic UWO241 constitutively maintains high ROS detoxification ability under all tested growth conditions. Constitutively unregulated activity of APX and GR in UWO241 suggests that the ascorbate-glutathione pathway is a major alternative electron sink to help maintain high photochemical activity and ROS detoxification in UWO241, especially in high salinity environments (Figures 2.10-2.13). Meanwhile, reduced activity in SAG49.72 suggests other ROS detoxification methods are more important. High levels of APX and GPX are common in extremophilic organisms. Tanaka et al. (2011) observed that *Chlamydomonas sp.* W80, a halophile, had a high level of APX activity comparable to that of psychrophilic UWO241. Similarly, they observed lower APX activity in mesophilic algae including *C. reinhardtii* (Tanaka et al. 2011). Additionally, *C. reinhardtii* had much higher catalase activity than *Chlamydomonas sp.* W80 (Tanaka et al. 2011). Thus it is possible that this H₂O₂ scavenging enzyme is more active in SAG49.72. Further activity assays of catalase, superoxide dismutase and glutathione peroxidase could provide additional information on ROS detoxification.

Immunoblotting revealed two isomers of APX in UWO241 that were approximately 5-7 kDa smaller (25 and 23 kDa) than SAG49.72 and other algae and higher plants (~30 kDa) (Fig. 2.11). Differences in apparent molecular mass of APX in UWO241 and SAG49.72 could be related to different requirements for membrane binding, which could impact catalytic activity (Chen and Asada 1989, Shigeoka et al. 2002). There are also different isoenzymes of APX present in the chloroplast, cytosol and peroxisomes. The antibody used for immunoblotting was not specific for a particular APX (i.e., chloroplast vs. cytosol APX), making it possible to observe multiple APX proteins of different sizes from a single sample. Additionally, multiple bands could be a cause of incomplete separation of membrane binding APX from the membrane. Further studies are required to determine if the differences observed in apparent molecular mass are associated with differences in enzyme activity. An APX activity gel assay was performed to determine which bands contained APX activity; however, the assay was not optimized for algae and was inconclusive (data not shown). Future research will involve optimization of APX gel activity assay along with rapid amplification of cDNA ends (RACE). RACE will provide a detailed analysis of the sequence, size and structure of the three APX cDNAs found in UWO241.
Another long-term acclimation mechanism present in UWO241 but absent in SAG49.72 was the induction of the osmoprotectant, glycerol, when exposed to high salinity environments. Increasing salinity increased production of glycerol through increased expression of glycerol synthase gene *serB* (Fig. 2.14). Osmoprotectants are essential for survival under high osmotic stress, which can be induced by high salt. Further research into *serB* and glycerol production could be beneficial for understanding osmoregulation in other algae and higher level plants under increasing salt stress.

Algal lipid bodies commonly accumulate in cultures exposed to extreme nutrient stress (Wang et al. 2009). However, our results provide new insights into increased lipid body accumulation under long-term abiotic stress acclimation. Our results also reveal UWO241 to be an organism with naturally high lipid content. Psychrophilic UWO241 contained much greater amounts of larger lipid bodies than SAG49.72. The total number of lipid bodies doubled when UWO241 was acclimated to low temperature conditions (Table 2.5). Increased lipid production combined with adaptation to extreme conditions makes UWO241 a great candidate for biofuel lipid production compared to less productive mesophilic organisms with limited growth conditions. Further research will expand on additional growth conditions and lipid analysis.

Our results reveal that *C. raudensis* UWO241 contains distinct and enhanced mechanisms for acclimation to long-term stress compared to its mesophilic relative, *C. raudensis* SAG49.72. UWO241 relies more heavily on ROS detoxification and protection, such as the ascorbate-glutathione pathway, than on photosynthetic modifications. In contrast, SAG49.72 acclimates to long-term stress with characteristic photosynthetic modifications, such as energy distribution between photosystems, altered Chl a/b ratio and increased RbcL for carbon fixation. Therefore, adaptation to extreme low temperature, low light and high salinity influence long-term stress acclimation between closely related strains.
Figure 2.1. Long-term stress treatment conditions for UWO241 and SAG49.72. Stress conditions were based on organisms’ specific physiology to induce similar long-term stress conditions. Cultures were inoculated from control conditions and exposed to long-term stress conditions until organisms became fully acclimated.
**Table 2.1.** Growth physiology parameters in cultures of UWO241 and SAG49.72 grown under control vs. long-term stress conditions. Values are means with S.D.s (n = 3 biological replicates).

a, statistical significance between control vs. stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05).

<table>
<thead>
<tr>
<th>Long-Term Stress Treatment</th>
<th>Photochemical ability (Fv/FM)</th>
<th>Total Chl (μg/mL)</th>
<th>Total Protein (μg/mL)</th>
<th>Total Chl/Total Protein (μg Chl/μg protein)</th>
<th>Chl/Cell (g Chl/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UWO241 Control</td>
<td>0.66 ± 0.02 (b)</td>
<td>6.99 ± 1.09</td>
<td>9.57 ± 0.23 (b)</td>
<td>0.73 ± 0.13</td>
<td>2.29 ± 0.53</td>
</tr>
<tr>
<td>UWO241 High Light</td>
<td>0.60 ± 0.01 (a)</td>
<td>4.02 ± 0.45 (a, b)</td>
<td>7.73 ± 1.81 (b)</td>
<td>1.04 ± 0.12 (a,b)</td>
<td>1.09 ± 0.09</td>
</tr>
<tr>
<td>UWO241 Low Temperature</td>
<td>0.55 ± 0.02 (a,b)</td>
<td>4.79 ± 0.59 (a)</td>
<td>4.63 ± 0.48 (a)</td>
<td>0.54 ± 0.15 (a)</td>
<td>1.48 ± 0.22 (b)</td>
</tr>
<tr>
<td>UWO241 High Salt</td>
<td>0.60 ± 0.05 (b)</td>
<td>5.06 ± 0.47</td>
<td>7.21 ± 1.57</td>
<td>0.73 ± 0.22</td>
<td>1.46 ± 0.14</td>
</tr>
<tr>
<td>SAG49.72 Control</td>
<td>0.70 ± 0.02 (b)</td>
<td>9.26 ± 1.62</td>
<td>2.50 ± 1.46 (b)</td>
<td>4.67 ± 0.36</td>
<td>1.58 ± 0.37</td>
</tr>
<tr>
<td>SAG49.72 High Light</td>
<td>0.65 ± 0.04</td>
<td>6.86 ± 0.08</td>
<td>3.66 ± 1.02 (a,b)</td>
<td>1.96 ± 0.50 (b)</td>
<td>1.03 ± 0.13</td>
</tr>
<tr>
<td>SAG49.72 Low Temperature</td>
<td>0.66 ± 0.01 (a)</td>
<td>4.31 ± 0.15 (a)</td>
<td>4.51 ± 1.87</td>
<td>1.07 ± 0.44</td>
<td>0.75 ± 0.049 (b)</td>
</tr>
<tr>
<td>SAG49.72 High Salt</td>
<td>0.66 ± 0.04 (b)</td>
<td>8.13 ± 0.84</td>
<td>3.40 ± 0.33</td>
<td>2.42 ± 0.47</td>
<td>1.44 ± 0.08</td>
</tr>
</tbody>
</table>
Figure 2.2. Maximum growth rates in cultures of UWO241 and SAG49.72 grown under control vs. long-term stress conditions. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with S.D.s (n = 3 biological replicates). a, statistical significance between control vs. stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05)
Figure 2.3. Ratios of Chl a/b in cultures of UWO241 and SAG49.72 grown under control vs. long-term stress conditions. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with S.D.s (n = 3 biological replicates). a, statistical significance between control vs. stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05)
Figure 2.4. Relative quantification of PsBA (A) and RubisCO (RbcL) (B) protein abundance in UWO241 and SAG49.72 grown under control vs. long-term stress conditions. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with S.D.s (n = 3 biological replicates). a, statistical significance between control vs. stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05)
Figure 2.5. Effect of long-term stress acclimation on photochemical quenching, qL (A,B) and energy partitioning of PSII (C,D) in UWO241 and SAG 49.72. qL, photochemical quenching \( \Phi \) NO, non-regulated energy dissipation; \( \Phi \) NPQ, non-photochemical energy quenching; \( \Phi \) PSII, PSII quantum yield. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with S.D.s (n = 3 biological replicates). a, statistical significance between control vs. stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05)
Figure 2.6. Effect of long-term stress acclimation on photosystem I activity determined by P700° in UWO241 and SAG 49.72. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means (n = 3 biological replicates).
**Table 2.2.** PSI activity determined by rate of oxidation in UWO241 and SAG49.72 acclimated to long-term stress conditions. Standard deviation presented of 3 replicates.

<table>
<thead>
<tr>
<th>Long-Term Stress Treatment</th>
<th>UWO241 Δ820nm</th>
<th>SAG49.72 Δ820nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0546 ± 0.0106</td>
<td>0.1048 ± 0.0099</td>
</tr>
<tr>
<td>High Light</td>
<td>ND</td>
<td>0.0363 ± 0.0027</td>
</tr>
<tr>
<td>Low Temperature</td>
<td>0.02483±0.0041</td>
<td>0.0519 ± 0.0022</td>
</tr>
<tr>
<td>High Salt</td>
<td>0.01706 ± 0.0114</td>
<td>0.1131 ± 0.0308</td>
</tr>
</tbody>
</table>
Figure 2.7. Effect of long-term stress acclimation on energy partitioning in photosystem I in UWO241 and SAG 49.72 grown under control vs. stress conditions. ΦPSI= quantum yield of photochemical energy to PSI. ΦND=Quantum yield of non-photochemical energy dissipation due to donor side limitation and ΦNA= acceptor side limitation. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with standard deviations (n = 3 biological replicates).
Figure 2.8. 77K fluorescence emission spectra and decomposition in Gaussian subbands of UWO241 (A,C,E,G) and SAG49.72 (B,D,F,H) acclimated to long-term stress treatments. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions).
Table 2.3. Summary of Gaussian fitting parameters for the subband decompositions of 77K Chl a fluorescence spectra in UWO241 and SAG49.72 cultures acclimated to control vs. long-term stress conditions.

<table>
<thead>
<tr>
<th>Long-Term Stress Treatment</th>
<th>UWO241</th>
<th>SAG49.72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LHCII/PSII</td>
<td>PSII/PSI</td>
</tr>
<tr>
<td>Control</td>
<td>1.89</td>
<td>2.23</td>
</tr>
<tr>
<td>High Light</td>
<td>2.05</td>
<td>3.86</td>
</tr>
<tr>
<td>Low Temperature</td>
<td>2.38</td>
<td>3.36</td>
</tr>
<tr>
<td>High Salt</td>
<td>0.93</td>
<td>113.97</td>
</tr>
</tbody>
</table>
Figure 2.9. The quantum yield of CEF to LEF in UWO241 and SAG49.72 acclimated to long-term stress treatments. ΦCEF/ΦPSII indicates the ratio of quantum yield of CEF to that of LEF. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with S.D.s (n = 3 biological replicates). a, statistical significance between control vs. stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05).
Figure 2.10. Relative quantification of ascorbate peroxidase protein abundance in UWO241 and SAG49.72 grown under control and long-term stress conditions. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with S.D.s (n = 3 biological replicates). a, statistical significance between control vs. stress condition within one algal strain (p < 0.05).
Figure 2.11. Representative immunoblot of ascorbate peroxidase isolated from UWO241 and SAG49.72 cultures grown under control conditions. SDS-PAGE samples were loaded on an equal protein basis of 5μg. The gel was probed with 1° anti-rabbit antibody raised against ascorbate peroxidase and 2° anti-rabbit antibody Protein A conjugated to horseradish peroxidase (HRP) (1:10,000 dilution). Numbers on the left represent the molecular mass markers (kDa).
Figure 2.12. Ascorbate peroxidase activity (µmol/min/µg protein) in UWO241 and SAG49.72 grown under control and long-term stress conditions. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with standard deviations (n = 3 biological replicates). b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05).
Figure 2.13. Total glutathione reductase activity per total protein (nmol/min/ng protein) of UWO241 and SAG49.72 grown under control vs. long-term stress conditions. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt (see Figure 1 for treatment descriptions). Values are means with S.D.s (n = 3 biological replicates). a, statistical significance between control vs. stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05).
Figure 2.14. Expression of \textit{serB} gene quantified by real time quantitative PCR across cultures of \textit{C. raudensis} UWO241 grown under variable salinity. A. Normalization of \textit{serB} functional gene expression using various reference genes. B. Expression of \textit{serB} normalized to 3 reference genes. C. Glycerol production measured by collaborator James Raymond at University of Nevada Las Vegas. Media salt concentration 10 mM, 300 mM, 700 mM and 1300 mM. Three technical replicates with S.D.s shown.
Table 2.4. Candidate reference genes and glycerol synthase gene identified from cDNA sequence library of *C. raudensis* UWO241. Primer pairs used in qPT-PCR at 52 °C.

<table>
<thead>
<tr>
<th>Contig #</th>
<th>Gene</th>
<th>Protein Name</th>
<th>Closets Match</th>
<th>% Identity</th>
<th>Primer Sequence</th>
<th>Size (bp)</th>
<th>Melting Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2262</td>
<td><em>rps10</em></td>
<td>Ribosomal protein S10</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>75.73</td>
<td>5’TGGTGTAGATGGGCCTCCCTG3’ 5’ACCCTCTTTCCGCCTTA3’</td>
<td>175</td>
<td>F: 53.7  R: 53.5</td>
</tr>
<tr>
<td>384</td>
<td><em>hist1h 2b</em></td>
<td>Histone H2B</td>
<td><em>Chlamydomonas sp. ICE-L</em></td>
<td>92.31</td>
<td>5’CCTTCATCAACGACATCT3’ 5’GGGAGAATGAGGCGGATT3’</td>
<td>117</td>
<td>F: 49.1  R: 54.5</td>
</tr>
<tr>
<td>444</td>
<td><em>gap3</em></td>
<td>GAPDH</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>81.13</td>
<td>5’TGACCGTGAACTCGCAGA3’ 5’TGACGAATGAGGCGGAGA3’</td>
<td>191</td>
<td>F: 55.5  R: 56.5</td>
</tr>
<tr>
<td>218</td>
<td><em>rpl19</em></td>
<td>Ribosomal protein L19</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>84.4</td>
<td>5’CGACCCCGAGAGGTAC3’ 5’ACAAGCAGAAGGCCAAGA3’</td>
<td>296</td>
<td>F: 53.4  R: 56.6</td>
</tr>
<tr>
<td>292</td>
<td><em>serB</em></td>
<td>Glycerol synthase</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>71.59</td>
<td>5’AGCGCAGAGACAACCA3’ 5’CATGGGCCTGTGTGTGAT3’</td>
<td>212</td>
<td>F: 55.3  R: 53.0</td>
</tr>
</tbody>
</table>
Figure 2.15. Total lipid bodies in UWO241 and SAG49.72 acclimated to long-term stress conditions. Lipids were stained with lipophilic fluorescent dye BODIPY 505/515 and observed under Zeiss Laser Scanning Microscopy (LSM 710) system. Lipid bodies are shown in green fluorescence and chlorophyll fluorescence in red.
Table 2.5. Quantification of total lipid bodies present, average lipid body radius, average lipid body volume and total volume lipid bodies per cell of UWO241 or SAG49.72 acclimated to long-term stress. Standard deviation shown from 10 replicates.

<table>
<thead>
<tr>
<th>Long-Term Stress Treatment</th>
<th>Lipid Bodies/Cell</th>
<th>Lipid Body Radius (µm)</th>
<th>Lipid Body Volume (µm^3)</th>
<th>Total Lipid Volume/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>UWO Control</td>
<td>12.9 ± 3.81</td>
<td>0.31 ± 0.05</td>
<td>0.17 ± 0.08</td>
<td>2.23 ± 1.20</td>
</tr>
<tr>
<td>UWO HL</td>
<td>15.7 ± 5.87</td>
<td>0.23 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.93 ± 0.45</td>
</tr>
<tr>
<td>UWO LT</td>
<td>21.9 ± 7.47</td>
<td>0.36 ± 0.10</td>
<td>0.29 ± 0.34</td>
<td>5.26 ± 3.72</td>
</tr>
<tr>
<td>UWO HS</td>
<td>12.3 ± 3.43</td>
<td>0.27 ± 0.04</td>
<td>0.10 ± 0.04</td>
<td>1.17 ± 0.37</td>
</tr>
<tr>
<td>SAG Control</td>
<td>7.4 ± 3.50</td>
<td>0.24 ± 0.08</td>
<td>0.10 ± 0.13</td>
<td>0.76 ± 1.07</td>
</tr>
<tr>
<td>SAG HL</td>
<td>26.9 ± 14.96</td>
<td>0.17 ± 0.09</td>
<td>0.04 ± 0.02</td>
<td>0.95 ± 0.64</td>
</tr>
<tr>
<td>SAG LT</td>
<td>8.7 ± 5.61</td>
<td>0.22 ± 0.09</td>
<td>0.08 ± 0.05</td>
<td>0.67 ± 0.55</td>
</tr>
<tr>
<td>SAG HS</td>
<td>11.7 ± 2.45</td>
<td>0.30 ± 0.06</td>
<td>0.15 ± 0.09</td>
<td>1.72 ± 0.83</td>
</tr>
</tbody>
</table>
CHAPTER THREE

CHARACTERIZATION OF SHORT-TERM PHOTOOXIDATIVE STRESS RESPONSE MECHANISMS IN PSYCHROPHILIC CHLAMYDOMONAS RAUDENSIS UWO241 COMPARED TO MESOPHILIC CHLAMYDOMONAS RAUDENSIS SAG49.72
3.1 INTRODUCTION

Most photosynthetic organisms must deal with short-term variability in their environments that occur over short time scales (from seconds to minutes), which can cause energy imbalances between energy generation (i.e., photochemical reactions) and energy consumed (i.e., downstream metabolic processes, including inorganic carbon fixation), and ultimately induce short-term photooxidative stress or photoinhibition. Organisms exposed to photooxidative stress induce a number of short-term stress responses, which require rapid induction, to prevent photooxidative damage (Dolhi et al. 2013, Davis et al. 2013). Short-term photoacclimatory mechanisms include state transitions, cyclic electron flow (CEF) around PSI, alternative electron transport pathways, the xanthophyll cycle, alteration of LHC proteins, as well as several energy dissipation mechanisms (Davis et al. 2013). These mechanisms are typical in mesophilic organism like *Chlamydomonas raudensis* SAG49.72. In marked contrast with most photosynthetic organisms, Antarctic lake algae such as *Chlamydomonas raudensis* UWO241 have evolved to survive long-term extreme environmental conditions; however, due to the permanent ice-cover on the Antarctic lakes, UWO241 are exposed to minimal environmental variability. Past studies have shown that UWO241 lacks several major short-term acclimatory mechanisms, including state transitions and alteration of LHC proteins (Morgan-Kiss et al. 2006, Pocock et al. 2011, Dolhi et al. 2013, Ivanov et al. 2012, Davis et al. 2013, Bonente et al. 2008).

The objective of this chapter was to test for differences in the abilities of closely related psychrophilic *C. raudensis* UWO241 and mesophilic *C. raudensis* SAG49.72 algae strains to respond to short-term low temperature and high light stress. Experiments will monitor: (i) short-term photoinhibition and recovery, and (ii) ROS production.
3.2 METHODS

3.2.1. Short-term stress treatments. Two short-term stress treatments were tested in cultures of C. raudensis UWO241 (P) and SAG49.72 (M). Short-term stress conditions were: (i) high light (HL) 8 °C/ 300 μmol m⁻² s⁻¹ (P) and 20 °C/ 300 μmol m⁻² s⁻¹ (M); and (ii) low temperature (LT) 2°C/ 50 μmol m⁻² s⁻¹. These conditions were chosen after testing sensitivity of both organisms to a range of light (50, 300 and 500 μmol m⁻² s⁻¹) and temperature (2 °C, 8 °C and 20 °C) treatments over three hours. UWO241 and SAG49.72 were grown under control conditions (8 °C, 50 μmol m⁻² s⁻¹ (P), 20 °C, 50 μmol m⁻² s⁻¹ (M)) and either BBM (M) or BBM/ 700 mM NaCl (P) until mid-log phase and then transferred to short-term stress condition for up to 1 hour. For experiments in which recovery from photoinhibition was monitored, following a short-term stress treatment, cultures were returned to control conditions and allowed to recover from the stress for 2 hours. Maximum quantum efficiency of PSII photochemistry (Fᵥ/Fₘ) and PSII energy partitioning (ΦPSII, PSII quantum yield; ΦNO, non-regulated energy dissipation; and ΦNPQ, non-photochemical energy quenching) were measured at regular time intervals using Chl a fluorescence as described in section 2.2.4. Percent photoinhibition was expressed as decrease of Fᵥ/Fₘ relative to control (time zero).

3.2.2. Immunoblotting of photosynthetic proteins: Immunoblotting was performed as described in section 2.2.6.

3.2.3. Quantitation of reactive oxygen species (ROS). Superoxide and H₂O₂ levels were quantified according Forster et al. (2005) with some modifications. Varying volumes of UWO241 or SAG49.72 mid-log phase cultures grown under conditions described above were transferred to dark tubes to achieve a total OD₇₅₀ = 2 (~12,500 cells) per sample. Twenty microliters of either 1 mM nitroblue tetrazolium (NBT; Sigma) or 5 mM 3,3’-diaminobenzidine-HCL (DAB; Sigma) dye was added to the cultures and incubated in the dark for 5 min to allow the dye to enter the cells. NBT reacts with superoxide and DAB reacts with H₂O₂. Following incubation, samples were filtered onto Whatman GF/C 25-mm filters and exposed to short-term stress (LT, 5°C, 50 μmol m⁻² s⁻¹; HL, 8°C or 20°C, 300 μmol m⁻² s⁻¹) for up to 1 hour. Following incubation, filters were immediately immersed in 80% acetone at zero (control), 30 or 60 min.
ROS levels were measured semi-quantitatively by densitometric analyses using the program ImageJ (http://imagej.nih.gov/ij/).

3.2.4. Chemical oxidant plate assays. Sensitivity to exogenously supplied chemical oxidants that produce various ROS was determined using an agar plate assay according to Baroli et al. (2003). Cultures grown to mid-log phase were serially diluted to achieve a range of total cells (10^5, 10^6, 10^7, 10^8) and spotted onto BBM plates containing variable concentrations of the oxidants rose bengal, methyl viologen, or H2O2. Ranges of concentrations of rose bengal (0.005 μM, 0.1 μM, 0.25 μM and 2.5 μM) were used to induce singlet oxygen, and methyl viologen (0.01 μM, 0.02 μM, 0.05 μM and 0.5 μM) were used to induce superoxide. H2O2 exposure was at 0.01 mM, 0.02 mM, 0.05 mM and 0.5 mM. Plates were incubated at either 10 °C (P) or 20 °C (M) and low light for 7 days. Colony growth was visually inspected daily to obtain a qualitative estimate of sensitivity to each of the oxidants.

3.2.5. Statistical analysis. Statistical analysis was performed as in section 2.2.11.

3.3 RESULTS

3.3.1. Short-Term stress photoinhibition. Sensitivity to temperature and irradiance levels was tested in UWO241 and SAG49.72 by exposing cultures to a particular stress and monitoring the decrease in FV/FM. Cultures were exposed to three irradiance levels (50, 300 and 500 μmol m^{-2} s^{-1}) or incubation temperatures (2 °C, 8 °C and 20 °C) for up to 3 hours. UWO241 exhibited high sensitivity to high light stress (300 and 500 μmol m^{-2} s^{-1}) regardless of incubation temperature. Three-hour irradiance exposure at 500 μmol m^{-2} s^{-1} resulted in 100% loss in FV/FM while exposure to 300 μmol m^{-2} s^{-1} resulted in ~50% photoinhibition in UWO241 at 8 °C (Fig. 3.1). In contrast, SAG49.72 was not affected by higher light levels but exhibited enhanced photoinhibition only at the low temperature. Low temperature stress induced approximately 50% photoinhibition of SAG49.72 after a 3-hour incubation (Fig. 3.1). Based on these initial experiments, 2 °C and 300 μmol m^{-2} s^{-1} were selected for further experiments on short-term temperature and light stress, respectively, as these conditions represented treatments under which the algae exhibited significant but not complete photoinhibition.
Cultures of UWO241 and SAG49.72 in mid-log phase were transferred from control to short-term stress conditions for 30 or 60 minutes. Exposure to high light short-term stress induced ~80% photoinhibition in the psychrophile UWO241, while the mesophile SAG49.72 experienced minimal changes in Fv/FM. Conversely, UWO241 experienced minimal photoinhibition when exposed to low temperature stress while SAG49.72 exhibited almost a 50% loss in Fv/FM following 1 hour of exposure to low temperature stress at 2 °C (Fig. 3.2). Thus, the psychrophile and mesophile strains exhibit differential sensitivity to high light and low temperature short-term photooxidative stress.

3.3.2. Recovery from short-term photoinhibition. Recovery from high light vs. low temperature short-term stress were measured for up to two hours in UWO241 and SAG49.72 cultures. Following 1 hr of low temperature or high light stress treatment, cultures were returned to control conditions to monitor the kinetics of short-term stress recovery. Despite differences in sensitivity to short-term photooxidative stress, both organisms exhibited full recovery within 120 minutes to initial Fv/FM levels once they were removed from the initial short-term stress (Fig. 3.2). These results indicate that the short-term stress-dependent loss in PSII photochemical efficiency was transitory and did not represent chronic damage to the photosynthetic apparatus. Despite rapid recovery of maximum PSII photochemical efficiency in both strains, there were strain- and treatment-specific differences at the level of the redox state (qL) as well as energy partitioning during the recovery phase. Low temperature and high light stress had minimal effects on these parameters in UWO241 and SAG49.72, respectively (Fig. 3.3). Short-term high light and low temperature stress in UWO241 and SAG49.72, respectively, dramatically reduced qL and ΦPSII. In addition the loss in ΦPSII was accompanied by increased thermal energy dissipation. UWO241 exposed to low temperatures experienced limited decrease in its qL, which was accompanied by reduced ΦPSII and a concomitant increase in quantum yield for non-photochemical quenching (ΦNPQ) (Fig. 3.3 B, D). UWO241 under all conditions had a higher capacity for ΦNO than SAG49.72 (Fig. 3.2 C, D). On the other hand, SAG49.72 exposed to low temperatures had a significant decrease in qL and increase in both ΦNPQ and ΦNO, while high light exposure increased ΦNO (Fig. 3.3 E-H). However, in almost all treatments, qL and ΦPSII returned to pre-stress levels during the recovery phase. One notable exception was UWO241 cultures exposed to short-term high light stress. High light-exposed samples of UWO241 were
unable to restore to initial qL or energy partitioning levels after 2 hours of recovery. In addition, \( \Phi_{\text{PSII}} \) remained low and induced major thermal dissipation through \( \Phi_{\text{NPQ}} \) while maintaining high \( \Phi_{\text{NO}} \) (Fig. 3.3 B, D).

3.3.3. *Immunoblotting of photosynthesis proteins.* Chronic photoinhibition can lead to alterations in the abundance of major photosynthetic proteins. Most significantly, the protein encoding the major subunit of PSII (PsbA) is extremely sensitive to photooxidative stress. Semiquantitative analyses of PsbA and RbcL polypeptide abundance via immunoblotting were chosen as indicators of possible chronic damage to components of the photosynthetic and carbon fixation pathways, respectively. Despite the sensitivity of PSII photochemical efficiency to photoinhibitory treatments, neither UWO241 nor SAG49.72 exhibited significant decreased in the PsbA PSII reaction center protein. Only UWO241 exposed to short-term high light stress experienced a statistically significant 20\% increase in RbcL abundance (Fig. 3.4).

3.3.5. *Production of reactive oxygen species:* Photooxidative stress is often accompanied by the production of ROS molecules. To test whether the psychrophilic and mesophilic algae produce ROS during high light- or low temperature-induced short-term stress, superoxide and hydrogen peroxide levels were measured before and after each stress treatment (Fig. 3.5). Incubating cultures with NBT (reacts with superoxide) or DAB (reacts with \( \text{H}_2\text{O}_2 \)) dyes provided semiquantification of cellular levels of ROS. SAG49.72 produced higher levels of superoxide after a 1-hour exposure to both high light (300 \( \mu\text{mol m}^{-2}\text{s}^{-1} \)) and low temperature (5 °C) short-term stress conditions relative to time zero, while UWO241 maintained very low levels of superoxide relative to SAG49.72 cultures, regardless of the treatment. \( \text{H}_2\text{O}_2 \) production was increased relative to the control in SAG49.72 cultures exposed to low temperature and in UWO241 cultures exposed to either high light or low temperature (Fig. 3.5).

3.3.6. *Chemical oxidant assay:* Since UWO241 and SAG49.72 exhibited differential levels of cellular ROS under short-term stress conditions (Fig. 3.5), we tested for sensitivity to exogenously supplied ROS using chemical oxidants in an agar plate assay (Fig. 3.6). In general, UWO241 exhibited higher sensitivity to all oxidants compared to SAG49.72 (Fig. 3.6). Both species exhibited the highest sensitivity to singlet oxygen production by rose bengal and
exhibited the lowest sensitivity to H$_2$O$_2$. Minimum oxidant concentration that induced sensitivity varied between organisms and type of oxidant. Both UWO241 and SAG49.72 were inhibited by the highest concentration of rose bengal (2.5 μM). UWO241 was more sensitive to H$_2$O$_2$ than SAG49.72 with inhibited growth at highest concentration (0.50 mM H$_2$O$_2$). UWO241 was particularly sensitive to superoxide produced by methyl viologen with inhibited growth at higher concentrations (0.05 and 0.5 μM) (Fig. 3.6).
3.4 DISCUSSION

In this chapter, short-term avoidance of acute stress was investigated in both organisms. Although the psychrophile, UWO241, exhibited a remarkable ability to acclimate to long-term stress, it possessed a more limited ability to respond to and recover from short-term oxidative stress compared to the mesophilic strain SAG49.72 (Figs. 3.2-3.3). These data support previous research that UWO241 is extremely sensitive to photoinhibition under higher light levels independent of incubation temperature (Pocock et al. 2007; Fig. 1). This apparent sensitivity to acute high light stress is likely a consequence of shade adaptation to its natural environment (< 50 μmol m⁻² s⁻¹), which has resulted in a relatively large PSII light harvesting complex (Neale and Priscu 1995, Morgan-Kiss et al. 2006). In contrast, SAG49.72 was not sensitive to high light but exhibited enhanced sensitivity to low temperature (Fig. 3.1-3.3). Being a mesophilic organism, SAG49.72 is much more sensitive to low temperatures and this short-term exposure to 2 ℃ can represent an extreme cold shock, comparable to low temperature shock in mesophilic crop plants.

The loss in photochemical activity in high light-treated UWO241 as well as low temperature-treated SAG49.72 was accompanied by a reduction in the PQ pool (i.e., decrease in qL; Figs. 3.2-3.3). Over-reduction of the PQ pool would lead to a decrease in linear electron flow, loss in photochemical performance, as well as increased the potential for ROS production and damage to photosynthetic proteins. However, excess excitation energy can be mitigated though non-photochemical quenching energy dissipation mechanisms. Because neither organism exhibited significant losses in PsbA PSII protein abundance under either stress condition, dissipation of excess energy largely accounted for mitigation of damage to PSII reaction centers in UWO241 and SAG49.72 (Fig. 3.4). Cultures of UWO241 at high light and low temperature dissipated excessive energy via both ΦNPQ and ΦNO, with a large emphasis on ΦNPQ (Fig. 3.3). Thermal dissipation was lower in SAG49.72 under both high light and low temperature stress (Fig. 3.3). Energy dissipation via ΦNPQ may involve a number of processes, including energy redistribution in favor of PSI excitation (i.e., state transitions) or heat dissipation via xanthophyll pigments (Pocock et al. 2007, Ivanov et al. 2012). In SAG49.72, state transitions accounted for a proportion of the induction of ΦNPQ, while the lack of state transitions in UWO241 suggests that xanthophyll pigments are likely to play a large role in energy dissipation.
in UWO241 (Pocock et al. 2007). Last, both organisms relied on inducible ΦNPQ as well as non-regulated alternative heat dissipation mechanisms ΦNO, but to different extents. Constitutively higher levels of ΦNO in UWO241 compared to SAG49.72 suggest the permanent need for non-regulated heat dissipation mechanisms such as CEF, ascorbate glutathione pathway and the plastoquinol terminal oxidase (PTOX) (Ivanov et al. 2012).

There are several lines of evidence that CEF plays a major role in energy dissipation in the psychrophilic UWO241 (Morgan-Kiss et al. 2002a, Chapter 2.3.6). Long-term stress acclimation analysis in Chapter 2 showed that CEF was higher in UWO241 than SAG49.72 under all stress conditions, while SAG49.72 potentially relied more on linear electron flow and alternative pathways, such as PTOX. CEF around PSI and PTOX alternative flow around PSII could help alleviate short-term increases of excitation pressure by cycling electrons instead of forming ROS. However, PTOX has been shown to produce superoxide in a side reaction (Heyno et al. 2009). Increased superoxide in SAG49.72 could be a product of PTOX (Fig. 3.6). The ascorbate glutathione pathway was shown to be constitutively upregulated under all growth conditions in UWO241 (Section 2.3.7) as a consequence of constitutively increased ascorbate peroxidase (APX) and glutathione reductase (GR) activity as well as constitutively increased ΦNO. Constitutively increased ΦNO was still detected under short-term stress conditions. Additional research is necessary to further determine the influence of the ascorbate-glutathione pathway as well as CEF in UWO241 and PTOX in SAG49.72 under short-terms stress.

Both organisms exhibited the ability to recover maximal photochemical activity under either high light or low temperature stress. Interestingly, the mesophilic SAG49.72 also exhibited recovery of the PQ oxidation state (i.e., qL); however high light-treated UWO241 maintained a highly reduced PQ pool even though photochemical ability completely recovered (Figs. 3.2-3.3). The inability to dissipate electrons from the PQ pool also explains why the psychrophile maintained a high level of ΦNPQ and ΦNO after recovery. Thus, while maximal photochemical efficiency completely recovered (Fig. 3.2), indicating minimal chronic damage to PSII protein PsbA (Fig. 3.4), the quantum yield of PSII and the PQ pool remained reduced in high light-treated cultures of the psychrophile (Fig. 3.3). This indicates that at least one or more processes responsible for oxidizing the PQ pool was inhibited under high light in UWO241, leaving the algae unable to recover in the time frame studied once the high light stress was removed. Although no damage to PSII PsbA protein was detected (Fig. 3.4), damage to other important
protein complexes present in the thylakoid could have been limiting recovery due to extreme high light-induced thylakoid acidification. These complexes are LHCII and LHCI, cyt b6f complex, oxygen-evolving complex (OEC), ATP synthase and NADPH dehydrogenase (NDH) (Jarvi et al. 2013). Acidification could disrupt the $\Delta \text{pH}$, causing high induction of $\Phi_{NPQ}$ and damage/inhibition of protein complexes. For example, the OEC, which is responsible for splitting water at PSII and releasing electrons, can become deactivated when pH is below 6.0 (Jarvi et al. 2013). Additionally, the cytochrome b$_6$f complex, which is responsible for the oxidation of the plastoquinol (PQH$_2$) and continues linear electron transfer, can become reduced due to low pH (Kramer et al. 1999). Last, proteases can assist in recovery by regulating the breakdown of other lumen proteins but may become deactivated under acidic conditions limiting recovery from short-term high light stress (Hall et al. 2010). Further research into thylakoid pH and additional protein complexes is necessary to fully understand the impacts of short-term high light stress.

ROS detection assays determined that UWO241 produces very low levels of superoxide but increased H$_2$O$_2$ under stress (Fig. 3.6). On the other hand, in SAG49.72 short-term stress increased superoxide production and allowed accumulation of relatively high levels of H$_2$O$_2$ (Fig. 3.6). These results suggest that UWO241 may have extremely active and efficient superoxide dismutase (SOD) that rapidly converts superoxide to H$_2$O$_2$ or that superoxide production is limited by low PSI activity in UWO241 (Morgan-Kiss et al. 2002a, Morgan et al. 1998, Section 2.3.5). Further chemical oxidant analysis revealed that UWO241 is extremely sensitive to superoxide, supporting the hypothesis that low superoxide production is due to decreased PSI activity (Fig. 3.7).

Exposure to chemical oxidants reveals resistance or sensitivity to specific ROS based on culture growth. Resistance would suggest that the organism contains mechanisms to mitigate and survive under the specific ROS, whereas sensitivity would suggest inability to mitigate ROS due to reduced exposure. Therefore, sensitivity to superoxide in UWO241 suggested reduced production of superoxide, while resistance to superoxide in SAG49.72 suggested highly active SOD. Both organisms showed high resistance to H$_2$O$_2$, corresponding with their high production (Fig. 3.6). High resistance to H$_2$O$_2$ in UWO241 could be an artifact of constitutively high ascorbate-glutathione pathway (Section 2.3.7). Due to limited superoxide production in UWO241, H$_2$O$_2$ is likely produced as a byproduct of photorespiration and not SOD; however,
additional research into photorespiration activity is necessary to determine influence on H$_2$O$_2$ production (Fig 3.7; Sirikhachornkit and Niyogi 2010, Apel and Hirt 2004, Hüner et al. 1998). Additionally, both species were sensitive to singlet oxygen, which is primarily produced at PSII. This suggests the potential presence of PTOX as an alternative electron sink at PSII to mitigate formation of singlet oxygen.

Our results revealed that *C. raudensis* UWO241 was more limited in its capacity to avoid and recover from short-term stress than *C. raudensis* SAG49.72. UWO241 was particularly sensitive to high light stress, which was likely due to extreme thylakoid acidification disrupting homeostasis and over-reduction of the PQ pool. Although both UWO241 and SAG49.72 relied on energy partitioning through thermal dissipation, SAG49.72 likely relied on ΦNPQ to induce state transitions while ΦNPQ in UWO241 induced the xanthophyll cycle. Lack of superoxide production and increased H$_2$O$_2$ production in UWO241 sparks interest for further research into production and detoxification of ROS.
**Figure 3.1.** Determination of sensitivities of UWO241 and SAG49.72 algae to short-term photooxidative stress. Cultures were grown under control conditions and exposed to either variable irradiance (LL = 50, ML = 300, HL = 500 μmol m$^{-2}$ s$^{-1}$) or temperature (2 °C, 8 °C, 20 °C). Photoinhibition was determined by decrease in PSII maximum photochemical efficiency ($F_V/F_M$) relative to time zero. S.D. shown, $n = 3$ biological replicates.
Figure 3.2. Percent photoinhibition in UWO241 and SAG49.72 induced during short-term stress conditions and recovery. Cultures grown under control conditions exposed to either high light (300 μmol m⁻² s⁻¹) or low temperature (2 °C) short-term stress for 60 min then returned to control conditions for 120 min to recover. S.D. shown, n=3 biological replicates. Percent photoinhibition was determined by decrease in photochemical ability (Fₖ/Fₘ) relative to time zero.
Figure 3.3. Effect of short-term stress response and recovery on photochemical quenching, qL (A,B,E,F) and energy partitioning of PSII (C,D,G,H) in UWO241 and SAG 49.72. Cultures were grown under control conditions then exposed to either high light (300 μmol m⁻² s⁻¹) or low temperature (2 °C) short-term stress conditions for 60 min then returned to control conditions for a 120 min recovery. qL, photochemical quenching ΦNO, non-regulated energy dissipation; ΦNPQ, non-photochemical energy quenching; ΦPSII, PSII quantum yield. Values are means with S.Ds (n = 3 biological replicates); a, statistical significance between control and stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05).
Figure 3.4: Relative quantification of PsbA (A) and RbcL (B) protein abundance in UWO241 and SAG49.72 exposed to high light (HL: 300 μmol m$^{-2}$ s$^{-1}$) and low temperature (LT: 2 °C) short-term stress over 1 hour. Values are means with standard deviations (n = 3 biological replicates); a, statistical significance between control and stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05).
Figure 3.5. Production of reactive oxygen species in UWO241 vs. SAG49.72 during short-term incubation in low temperature (5 °C) or high light (300 μmol m⁻² s⁻¹) stress. Algal samples were incubated for 1 hour with NBT or DAB stain to detect superoxide or hydrogen peroxide production respectively. (n = 3 biological replicates); a, statistical significance between control and stress within one algal strain; b, statistical significance between UWO241 and SAG49.72 when grown under the same condition (p < 0.05).
**Figure 3.6.** Sensitivity of UWO241 (A) and SAG49.72 (B) to varying concentrations of chemical oxidants that result in production of different ROS molecules. Algae were spotted onto agar plates containing varying concentrations of chemical oxidants that induce ROS and grown in the incubator for 7 days. Hydrogen peroxide [0.1 mM, 0.02 mM, 0.05 mM and 0.5 mM] provides H$_2$O$_2$ while rose bengal [0.005 μM, 0.1 μM, 0.25 μM and 2.5 μM] and methyl viologen [0.01 μM, 0.02 μM, 0.05 μM and 0.5 μM] produce singlet oxygen and superoxide, respectively. Varying cell density ($10^5$, $10^6$, $10^7$, $10^8$) determined differential sensitivity. Spot intensity reflects cell growth and resistance to chemical oxidant.
CHAPTER 4

4.1 CONCLUSION AND FUTURE DIRECTIONS

The vast majority of photosynthetic organisms are exposed to a variety of changing environmental conditions that can vary across broad time scales. Some of these conditions are non-deleterious and can lead to improved survival or colonization of new environmental niches; however, exposure to excessive stress conditions can cause deleterious effects on an organism’s survival. A common outcome of exposure to high environmental stress (e.g., high light, low temperatures, high salinity, nutrient deprivation) in photosynthetic organisms is the production of reactive oxygen species (ROS) molecules that can lead to extensive cellular damage. Thus, photosynthetic organisms need to maintain a variety of oxidative stress response mechanisms to respond to and ultimately survive in a changing environment. These mechanisms include limiting production of ROS as well as destruction of ROS through scavenging pathways. Algae are single-celled photosynthetic organisms, which have been exploited as simple photosynthetic models for studying environmental stress response. However, to date only a small number of model algal species have been thoroughly studied and all of these organisms have evolved under relatively moderate environments. A unique subset of photosynthetic organisms adapted to environmental stress of permanent low temperatures are the psychrophilic algae, or “photopsychrophiles”. This thesis focused on how psychrophilic in comparison with mesophilic photosynthetic organisms respond and adapt to short- and long-term stress exposure. Findings from this research could be important for future applications for improvement of crop stress tolerance by discovery of novel photooxidative stress avoidance pathways in an organism which is permanently exposed to high environmental stress (e.g., low temperatures, high salinity). In addition, psychrophilic algae could also harbor novel biotechnological applications, including the ability to produce and store high oil content.

The research presented here expands upon current knowledge of photooxidative stress in organisms adapted to permanent cold environments by investigating long-term (Chapter 2) and short-term (Chapter 3) stress response mechanisms in a psychrophilic green alga, *Chlamydomonas raudensis* UWO241, compared to a mesophilic relative, *Chlamydomonas raudensis* SAG49.72. This thesis both supported previous findings regarding environmental acclimation in photopsychrophiles as well as revealed new stress response mechanisms. Our
results supported previous findings that UWO241 lacked the ability to utilize typical photosynthetic modifications during long- and short-term stress, such as the ability to alter LHCII size and modulation of energy distribution via state transitions (Pocock et al. 2011, Dolhi et al. 2013, Wilson et al. 2006, Morgan-Kiss et al. 2002a, Ivanov et al. 2012, Davis et al. 2013). These results also support and expand on research regarding differential use of mechanisms of thermal dissipation of excess energy in UWO241 and SAG49.72. Past reports observed that SAG49.72 relied more heavily on inducible non-photochemical quenching (ΦNPQ) associated with the xanthophyll cycle and antenna quenching, while UWO241 appeared to utilize the less understood non-regulated energy quenching (ΦNO) for survival under high temperature acclimation (Szyszka et al. 2007). Our results revealed that while both organisms rely on ΦNPQ under certain long- and short-term stress conditions, UWO241 also maintains constitutively high capacity for ΦNO regardless of the growth condition or treatment, relative to SAG49.72. Previous work also noted high ΦNO in UWO241; however, the underlying mechanisms were not understood. In this present study, we suggest that higher ΦNO activity in UWO241 may be associated with alternative electron sink pathways, such as cyclic electron flow (CEF) and the ascorbate-glutathione pathway (ASH-GSH pathway).

UWO241 appears to have novel adaptations to deal with a natural habitat that is permanently under high oxidative stress. For example, the ASH-GSH pathway appears to be permanently upregulated in this organism. This was supported by upregulated ascorbate peroxidase (APX) and glutathione reductase (GR) enzyme activity results. In addition, altered apparent molecular mass of APX protein in UWO241 may indicate a unique psychrophilic APX isoenzyme. The altered apparent molecular mass could be related to membrane binding requirements and catalytic activity. Additional analysis of APX through rapid amplification of cDNA ends (RACE) will provide a detailed analysis and comparison of the amino acid sequence and structure of the APX found in extremophilic UWO241 to other organisms. Additional research into other ROS scavenging enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) could provide insight into other ROS defense mechanisms present in UWO241 compared to SAG49.72. Mesophilic algae, similar to SAG49.72, have higher CAT activity with reduced APX activity, further suggesting a potential for increased CAT in SAG49.72 (Tanaka et al. 2011). CAT, SOD and GPX activity can be determined by colorimetric assays as in Tanaka et al. 2011.
In addition to ROS detoxification, avoidance of ROS production through mechanisms that maintain redox poise and photostasis under photooxidative stress have been observed across many taxonomically diverse photosynthetic organisms. The plastoquinol terminal oxidase (PTOX) may be another alternative electron sink present in both UWO241 and SAG49.72. PTOX is present at PSII and acts as an alternative sink, capturing electrons and forming water instead of ROS (Heyno et al. 2009). Two putative PTOX genes have been identified from the transcriptome of UWO241, suggesting the need for additional research. An early experiment would test for the presence of PTOX-like proteins in both organisms via immunoblotting. The application of appropriate inhibitors that can prevent electron flow through carbon fixation, photorespiration, CEF and the ASH-GSH pathway will allow determination of the ability of PTOX to maintain electron flow and PQ pool reoxidation (Laureau et al. 2013).

UWO241 is a new model for photoacclimation in extremophilic organisms but may also provide new insights for understanding alternative induction of lipid production relative to other model algal species. We have discovered that UWO241 exhibits constitutively higher lipid production than SAG49.72, particularly under low temperature stress. This is a remarkable discovery, considering that all other studied algae to date require a treatment of extreme nutrient stress to induce significant accumulation of storage lipids. Essentially, algae such as the model C. reinhardtii stop growing under nutrient stress, degrade their chloroplasts, and convert photosynthetic membranes into storage lipids (Msanne et al. 2012, Scott et al. 2012, Merchant et al. 2012). Our results indicate that UWO241 synthesizes storage lipids de novo while maintaining high growth rates and photosynthetic activity. Thus, UWO241 may be a novel organism for understanding how an algal species can accumulate high oil while actively growing. Future studies are necessary to better understand the biosynthetic pathway for neutral lipid production in UWO241.

Although UWO241 had distinct and enhanced mechanisms for adaptation to long-term stress, it also exhibited extreme sensitivity to short-term high light stress compared to SAG49.72. Interestingly, despite this sensitivity, both organisms exhibited full recovery of PSII photochemical efficiency (Fv/Fm) within a short period of time, indicating that the sensitivity exhibited by UWO241 was not chronic (e.g., no damage to PSII reaction center proteins). Both organisms also exhibited comparable levels of H2O2 production under short-term stress, while superoxide production was significantly higher in SAG49.72 relative to UWO241. Low
superoxide production in UWO241 corresponds with low PSI activity and high sensitivity to methyl viologens. Thus, H$_2$O$_2$ in UWO241 is likely a product of photorespiration, not SOD. Further analysis and optimization of filter dye assay may be necessary to more accurately determine H$_2$O$_2$ production.

Despite recovery of F$_V$/F$_M$, UWO241 exposed to short-term high light stress exhibited minimal recovery of other parameters (qL, $\Phi$NPQ, $\Phi$NO). Limited recovery was likely due to acidification of the thylakoid, potentially disrupting the $\Delta$pH, ATP synthases, $\Phi$NPQ and other photosynthetic proteins besides PsbA, such as the oxygen evolving complex or the cytochrome b$_6$f complex (Jarvi et al. 2013, Kramer et al. 1999). Additional studies of the organisms under short-term stress are necessary to fully determine and characterize short-term response mechanisms. Of particular interest is measuring the trans-thylakoid pH. This can be accomplished through the use of pH-indicating probes such as 9-amino-acridine; however, measuring an accurate thylakoid pH can be very difficult (Tikhonov et al. 2008, Kramer et al. 1999).

4.2 MODEL OF PHOTOACCLIMATION

This study provided additional information regarding acclimation to long-term photooxidative stress, and production of a new comparative model for photoacclimation between a cold-adapted photosynthetic organism, *C. raudensis* UWO241, and its close mesophilic relative, *C. raudensis* SAG49.72 (Fig. 4.1). During growth under control conditions (i.e., under a non-stress or optimal growth regime), the PQ pool is relatively oxidized in both organisms. Linear electron transport is favored because the rate of consumption of photosynthetic electrons through metabolic sinks, such as carbon fixation and photorespiration, is balanced with the rate of water splitting at PSII (Fig. 4.1A). Even under these control conditions this is quite remarkable, considering that UWO241 is growing at growth temperatures far below the minimum growth temperature for SAG49.72. When UWO241 (Fig. 4.1B) or SAG49.72 (Fig. 4.1C) are exposed to an environmental stress (i.e., high light, low temperature or high salinity), LEF can become disrupted due to over-reduction of the PQ pool caused by over-excitation of PSII or decreased availability of electron acceptors at PSI (NADP$^+$). Once electron sinks within the photosynthetic electron transport chain become unavailable, excess electrons and/or excited
chlorophylls would begin to react with molecular oxygen to form ROS molecules (i.e. superoxide (O$_2^-$), singlet oxygen (¹⁰₂*) and H$_2$O$_2$; Fig. 4.1B,C).

Two types of photoacclimation mechanisms exist to avoid the accumulation of ROS under photooxidative stress: (i) photosynthetic modifications to maintain redox poise/photostasis and therefore avoid production of ROS and (ii) ROS detoxification via enzyme- and non-enzyme-catalyzed pathways. Our data suggest that UWO241 and SAG49.72 rely on some similar and some different mechanisms for photoacclimation. Although energy partitioning (i.e., redistribution of excitation energy from photochemical reactions – ΦPSII – to energy dissipative mechanisms – ΦNPQ and/or ΦNO) is important for survival in both organisms under long-term stress, they each induced different mechanisms. ΦNPQ was particularly important under high light stress in both strains, likely inducing energy distribution between photosystems to favor PSI (state transitions) and the xanthophyll cycle in SAG49.72. Since state transitions are absent in UWO241, development of ΦNPQ is likely solely dependent upon the xanthophyll cycle.

Additionally SAG49.72 relied more on photosynthetic modification to avoid ROS production such as modifications of photosynthetic apparatus (e.g., loss of Chl b suggesting reduced LHCII size under high excitation pressure) and upregulation of RbcL for carbon fixation under high light stress. The ability of SAG49.72 to increase growth rates in combination with upregulation of RbcL abundance under high light stress indicates that the mesophile controls excess electron excitation by increase carbon fixation. Our results suggest that during photoacclimation UWO241 cannot rely on typical modifications to the photosynthetic apparatus (i.e., UWO241 had a constitutively lower non-adjustable Chl a/b ratio with a permanently large LHCII and reduced PSI activity). However, constitutively higher levels of thermal dissipation through ΦNO in UWO241 suggested increased energy partitioning through alternative transport pathways to help maintain redox poise and a relatively oxidized PQ pool. There are two main alternative transport pathways permanently upregulated in UWO241 under control conditions, CEF and the ASH-GSH pathway, which can further improve survival under numerous long-term environmental stresses. PTOX may also potentially act as an alternative electron sink at PSII in both organisms suggested by presence of donor side limitation; however, additional research is necessary to establish this paradigm. In addition, under all growth conditions UWO241 relied on APX to detoxify H$_2$O$_2$. Although SAG49.72 may induce APX under high salt conditions, literature for mesophilic algae, such as SAG49.72, suggests that CAT plays a prominent role in
ROS defense (Tanaka et al. 2011). Many long-term stress acclimation mechanisms remain uncertain, but this thesis provides a basis for future studies of stress avoidance or stress-mitigating mechanisms in a psychrophilic and a mesophilic strain of *C. raudensis*. Future studies can include lipid production for biofuels or improving tolerance to environmental stress.
Figure 4.1. A simplified overview of long-term photooxidative stress response mechanisms in *C. raudensis* UWO241 and SAG49.72 during photoacclimation to control, high light, low temperature and high salt stress conditions. The model shows possible energy partitioning of absorbed light energy into photosynthesis ($\Phi_{\text{PSII}}$; blue arrow), thermally dissipated via $\Delta\text{pH}$ and xanthophyll-dependent energy quenching ($\Phi_{\text{NPQ}}$, red arrows) or dissipated through $\Phi_{\text{NO}}$ alternative electron sinks (dotted lines). (A) Under control conditions, linear electron flow (LEF) of photosynthetic electrons from PSII to PSI into metabolic sinks such as carbon fixation and photorespiration dominates. (B,C) Environmental stressors such as high light and low temperature can overexcite PSII and disrupt the regeneration of NADP$^+$, respectively. This can disrupt LEF, causing the PQ pool to become over-reduced, increasing excitation pressure and the formation of ROS ($^{1}\text{O}_2$, $\text{O}_2^{-}$, $\text{H}_2\text{O}_2$; shown in red). Different environmental stressors can induce different energy partitioning or ROS defense mechanisms as shown by specified bold color: control (black), high light (orange), low temperature (light blue) and high salt (pink). (B) Under all conditions, UWO241 maintains a much larger LHCII and greatly reduced PSI activity. UWO241 also relies heavily on the ascorbate-glutathione pathway (ASH-GSH pathway) for both reduction of excitation energy and ROS detoxification. CEF is upregulated under high light and low temperature along with $\Phi_{\text{NPQ}}$ under high light. The ASH-GSH pathway is additionally upregulated under high salt. High light and high salt stress can potentially induce PTOX. (C) Under all conditions, SAG49.72 maintains LEF by having high PSI activity with reduced LHCII size and PSII reaction center abundance. High light stress induces $\Phi_{\text{NPQ}}$ as well as $\text{CO}_2$ fixation and potentially PTOX, while high salt potentially increases APX and PTOX activity. CAT likely acts as the main ROS detoxification enzyme in SAG49.72, as opposed to APX in UWO241.
REFERENCES


Haines J, Kelley M. 2010. Demonstration of a ΔΔCq calculation method to compute the relative gene expression from qPCR data. Thermo Scientific Tech Notes.


alternative oxidase (PTOX) promotes oxidative stress when overexpressed in Tobacco. Metabolism and Bioenergetics. 284(45): 31174-31180


Kong W, Nakatsu CH. 2010. Optimization of RNA extraction for PCR quantification of aromatic compound degradation genes. Applied and Environmental Microbiology. 76: 1282-1284


Steig EJ, Schneider DP, Rutherford SD, Mann ME, Comiso JC, Shindel DT. 2009.


Vincent WF. 2010. Microbial ecosystem response to rapid climate change in the Arctic. ISME Journal. 4, 1089-1091.

