Cyclic electron flow and ascorbate pathway play a role in survival of Chlamydomonas sp. UWO241 to long-term photooxidative stress

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Abstract

Under environmental stress plants and algae employ a variety of strategies to avoid damage to the photosynthetic apparatus and maintain photosynthetic capacity. To date, most studies on stress acclimation have focused on model organisms possessing limited tolerance to elevated stress levels. We compared the long-term acclimatory capacities of a mesophilic alga (Chlamydomonas raudensis SAG 49.72; SAG 49.72) and an Antarctic halotolerant psychrophile (Chlamydomonas sp. UWO 241; UWO 241) by monitoring photobiology, cyclic electron flow (CEF) and ROS defense in cultures acclimated to long-term low temperature, high salinity or high light stress. SAG 49.72 responded to long-term stress by increasing chlorophyll a/b ratio and redistributing absorbed light energy from photosystem II (PSII) to photosystem I (PSI). In contrast, the psychrophile exhibited faster half times for P700+ re-reduction under all treatments, suggesting high CEF rates. High CEF was accompanied by increased capacity for nonphotochemical quenching. Last, UWO 241 exhibited constitutively high activity of two key ascorbate cycle enzymes, ascorbate peroxidase and glutathione reductase, as well as a large ascorbate pool. Our results suggest that UWO 241 relies on high PSI-mediated CEF and ROS detoxification to protect the photosynthetic apparatus while minimizing energy expenditure on repairs.

INTRODUCTION

Photostasis is a phenomenon common to all photosynthetic organisms: it encompasses processes which contribute to balancing rates of photosynthetic energy absorbed with energy consumed by metabolism (Öquist & Hüner, 2003). Disruption of photostasis is manifested as an accumulation of a reduced pool of the mobile electron acceptor, plastoquinone (PQ), leading to photooxidative stress. This phenomenon occurs under excessive light conditions; however, any environmental condition which impacts an organism's ability to use absorbed light energy can lead to an over-reduction of the PQ pool (Hüner et al., 2012; Morgan-Kiss, Priscu, Pocock, Gudynaite-Savitch, & Hüner, 2006). Thus, any alteration in an organism's environment can exacerbate disruption to photostasis and enhance the probability of photooxidative stress, including day/night cycle, salinity, drought, heat, chilling, and nutrient status (Bartels & Sunkar, 2005; Ensminger, Busch, & Hüner, 2006; Liu, Qi, & Li, 2012; Sharma, Jha, Dubey, & Pessarakli, 2012; Takahashi & Murata, 2008).

A major biproduct of unbalanced photosynthesis is the production of reactive oxygen species (ROS). ROS accumulates when the photosynthetic electron transport chain becomes over-reduced, causing oxidative injury and damage to proteins, lipids, nucleic acids, and many components of the photosynthetic apparatus (Apel & Hirt, 2004; Asada, 1996; Møller, Jensen, & Hansson, 2007; Sirikhachornkit & Niyogi, 2010). Oxidative stress responses 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 (Niyogi, 1999; Suzuki, Koussevitzky, Mittler, & Miller, 2012). Short-term responses are non-heritable adjustments to physiology and biochemistry which avoid ROS production (Ledford, Chin, & Niyogi,

2007; Sirikhachornkit & Niyogi, 2010). Common short-term stress response mechanisms are phototaxis, state transitions, nonphotochemical quenching (NPQ), and alternative electron transport pathways, such as the water-water cycle and PSI-associated CEF (Kozi Asada, 2000; Cournac et al., 2002; Minagawa, 2011; Müller, Li, & Niyogi, 2001; Witman, 1993). Changes in gene expression and protein translation aid in maintenance of photostasis over longer time scales. Long-term responses can involve minimizing ROS production and/or increasing ROS detoxification, and include changes to antenna size or PSI/PSII stoichiometry increased CO_2 fixation capacity, and activation of antioxidant pathways (Kozi Asada, 2006; Falk et al., 1994; Falk, Maxwell, Gray, Rezansoff, & Hüner, 1993; Lucker & Kramer, 2013; Tanaka & Melis, 1997; Yamori, Makino, & Shikanai, 2016). Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and enzymes of the ascorbate-glutathione (AsA-GSH) cycle (Noctor & Foyer, 1998). Maintenance of high antioxidant capacity has been associated with tolerance to environmental stress in plants and algae (Aldesuquy, Baka, El-Shehaby, & Ghanem, 2013; Chen, Zhang, & Shen, 2011; Van Alstyne, Sutton, & Gifford, 2020). The metabolite ascorbate is particularly versatile in ROS defense, possessing the ability to directly react with several ROS molecules, including singlet oxygen, superoxide and hydroxyl radicals. Plants accumulate millimolar levels of ascorbate; however, algae and cyanobacteria contain significantly lower levels of the antioxidant (Foyer & Halliwell, 1976; Foyer, Lopez-Delgado, Dat, & Scott, 1997; Hu et al., 2008).

Some photosynthetic organisms have evolved to survive and grow in permanently stressful environments. Relative to the well-studied processes of short- and long-term stress acclimation, strategies of photosynthetic adaptation to permanent abiotic stress are significantly less understood. Low temperature environments are abundant at high latitudes (Young & Schmidt, 2020): photopsychrophiles are photosynthetic organisms which are physiologically adapted to permanent low temperatures (Morgan-Kiss et al., 2006). The Antarctic *Chlamydomonas* sp. UWO 241 (UWO 241) was isolated from a permanently ice-covered, hypersaline lake and represents one of the few models for photosynthetic adaptation to combined low temperatures and high salinity (Cvetkovska, Hüner, & Smith, 2017). Early studies reported that UWO 241 exhibits minimal capacity for short-term acclimatory mechanisms, such as the xanthophyll cycle and state transitions (Morgan-Kiss, Ivanov, & Hüner, 2002; Morgan, Ivanov, Priscu, Maxwell, & Hüner, 1998), and sensitivity to short-term thermal or high light stress (Morgan-Kiss, Ivanov, Williams, Mobashsher, & Hüner, 2002; T. Pocock, Koziak, Rosso, Falk, & Hüner, 2007). In lieu of short-term acclimation, UWO 241 has evolved to rely on constitutive mechanisms as a consequence of adaptation to permanent low temperatures and high salinity (Morgan-Kiss et al., 2006). While UWO 241 exhibits high susceptibility to high light stress, it also possesses the ability to rapidly recover from photoinhibition (Pocock et al., 2007). Despite the presence of cold-active thylakoid kinases, energy transfer from PSII to PSI uses a poorly understood spill-over mechanism (Szyszka-Mroz et al., 2019). In addition, under native low temperature and high salinity conditions, UWO 241 assembles a novel PSI supercomplex which allows the organism to maintain a strong capacity for PSI-driven CEF (Cook et al., 2019; Szyszka-Mroz, Pittock, Ivanov, Lajoie, & Hüner, 2015). The additional proton motive force (pmf) derived from CEF is used for constitutive capacity for NPQ and production of additional ATP in cells grown under high salinity (Kalra et al., 2020). High ATP production may suggest a lower requirement for reductant under permanent environmental stress where growth and assimilation rates could be slow. The adjustments to the photosynthetic apparatus are accompanied by alterations in carbon metabolism, including upregulation of several enzymes within the Calvin Benson Bassham cycle (CBB), and key enzymes of the shikimate pathway, a high carbon flux pathway which synthesizes precursors for aromatic metabolites (Julkowska, 2020; Kalra et al., 2020). Together, these novel adaptive strategies allow UWO 241 to maintain robust growth and photosynthesis under the combined stress of permanent low temperature and high salinity.

While activation of CEF is known to be essential in plants and algae exposed to short-term stress, the discovery of a strong CEF capacity in a psychrophilic, halotolerant alga suggests that there is a previously unappreciated role for CEF during long-term adaptation to environmental stress. We hypothesized that constitutive CEF and ROS detoxification allows UWO 241 to survive multiple stresses by maintaining photostasis and protecting the photosynthetic apparatus from photooxidative damage. We tested this hypothesis by comparing growth physiology as well as PSII and PSI photochemistry in UWO 241 and a related meso-

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philic species, *Chlamydomonas raudensis* SAG 49.72, acclimated to long-term stress conditions (high light, low temperature, high salinity). We also monitored production of a major ROS (O_2^-) as well as activity of two key enzymes of the AsA-GSH pathway (Ascorbate Peroxidase, APX; Glutathione Reductase, GR) and levels of AsA-GSH substrate, ascorbate. Our study shows that UWO 241 possesses robust ability to acclimate to long-term photooxidative stress by both avoiding ROS production by maintaining photostasis through CEF and relying on constitutive ROS detoxification. We suggest that this reliance on the redundant systems allow the organism to withstand long-term exposure to multiple stressors in its native habitat while minimizing energy expenditures for repair processes.

MATERIALS AND METHODS

Strains, growth conditions and growth physiology

Cultures of the psychrophilic *Chlamydomonas* sp. UWO 241 (CCMP1619) and a mesophilic strain, *Chlamydomonas raudensis* SAG 49.72, were grown in Bold's basal medium (BBM) (Nichols & Bold, 1965) under ambient CO_2 conditions in 250 mL Pyrex tubes submerged in temperature-regulated aquaria as described in Morgan-Kiss et al. (2008). The mesophilic SAG 49.72 was chosen for comparison as it has been used in several comparative studies with UWO 241 (Pocock, Vetterli, & Falk, 2011; Szyszka-Mroz et al., 2019; Szyszka-Mroz et al., 2015; Szyszka, Ivanov, & Hüner, 2007). Cultures were grown under either control (C) conditions or exposed to one of three long-term stress treatments: high light (HL), low temperature (LT) or high salt (HS). For control conditions, cultures were grown under temperature/light regimes of 8°C/50 μmol $^{-2}s^{-1}$ and 20°C/50 μmol^{-2} s^{-1} for UWO 241 and SAG 49.72, respectively, and NaCl levels of 0.43 mM for both strains (Table 1). Long-term stress conditions were chosen based on previous studies (Morgan-Kiss et al., 2006; Pocock et al., 2011; Szyszka et al., 2007) to reflect the maximum level of a particular stress to which the organism could fully acclimate, that is achieve exponential growth and high photochemical activity (maximum photosynthetic efficiency values, F_V/F_M, above 0.5). Long-term stress conditions for UWO 241 and SAG 49.72, respectively, were: i) high light, 250 μmol m^{-2} s^{-1} and 500 μmol m^{-2} s^{-1}; ii) low temperature, 2° C and 11 ° C; iii) high salinity, 700 mM NaCl and 100 mM NaCl.

Growth kinetics were monitored as change in optical density at 750 nm. All other measurements were performed on mid-log phase cultures. Chlorophyll a and b concentrations were determined from whole cell extractions in 90% acetone according to Jeffry & Humphrey (1975).

Room temperature chlorophyll fluorescence

The activities of PSI and PSII were measured in dark-adapted (10 min) exponentially growing cultures with a Dual-PAM-100 system (Heinz Walz GmbH, Effeltrich, Germany) as described in Szyska et al. (2007). All samples were supplemented with 10 mM sodium bicarbonate and measurements were performed in a water-jacketed cuvette at the corresponding growth temperatures. The fluorescence parameters F_V/F_M (maximum photochemical efficiency), qL (proportion of open PSII reaction centers, assuming the "lake" model for antenna connectivity between reaction centers, qL=qP · [Fo'/FS]), Φ (PSII) (quantum yield of photochemistry, ΦPSII=(Fm ' - Fs) /Fm',), and NPQ (nonphotochemical energy dissipation from antenna quenching - Fm-Fm ')/Fm', were calculated during steady state photosynthesis (Kramer et al. 2004). Energy partition parameters for PSII were: ΦNPQ (ΦNPQ=1-ΦPSII-ΦNO) and ΦNO (energy dissipation from non-regulated processes- ΦNO=1/[NPQ+1+qL (Fm/Fo-1)]). Energy partition parameters of PSI were: quantum yield of PSI (ΦPSI), acceptor side limitation (ΦNA), and donor side limitation (ΦND) (Schreiber & Klughammer, 2008). All measurements were performed under temperature and irradiance values which matched the growth conditions.

Low temperature Chl a fluorescence (77K)

Low temperature (77 K) Chl fluorescence emission spectra were measured using a Perkin Elmer Luminescence Spectrometer (LS50B) (Buckinghamshire, England) equipped with liquid nitrogen accessory. Algal cultures from dark-adapted (10 min) mid-log phase cultures were transferred to NMR tubes and flash frozen in liquid nitrogen. Fluorescence spectra were collected at the excitation wavelength of 435 nm and recorded at a slit

width of 4 nm for excitation and emission. 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. (2002b) using the program OriginPro 8.5.1.

P700 reduction/oxidation kinetics

Far red or red light induced photooxidation of P700 was used to determine rates of CEF as described by Morgan-Kiss et al. (2002b). A volume of exponential phase cultures representing 25 µg Chl a was dark adapted for 10 min and then filtered onto 25 mm GF/C filters (Whatman, Cat No. 1822-025). Filters were measured on the Dual-PAM 100 instrument using the leaf attachment. The proportion of photooxidizable P700 was determined by monitoring absorbance changes at 820 nm and expressed as the parameter ($[?]A_{820}/A_{820}$). The signal was balanced and the measuring light switched on. Far red (FR) light ($\lambda max=715$ nm, 10 Wm^{-2}, Scott filter RG 715) was then switched on to oxidize P700. For the red light measurement, cells were pre-treated in the dark for 10 min in the presence of 10 µM DCMU to inhibit electron transfer from PSII to PSI. After steady-state oxidation levels were reached, the FR or red light was switched off to re-reduce P700. The half time for the reduction of P700$^+$ to P700 ($t_{\frac{1}{2}}^{red}$) was calculated as an estimate of relative rates of PSI-driven CEF (Ivanov, Morgan, Gray, Velitchkova, & Hüner, 1998). The re-reduction time for P700 was calculated using the program OriginPro 8.5.1 using first order exponential decay kinetics.

Ascorbate and AsA-GSH Pathway

Glutathione reductase (GR) activity was measured using a glutathione reductase assay kit based on NADPH oxidations (Kit 703202, Cayman Chemicals, Ann Arbor). Mid-log phase cultures (~3-10 x 10^6 cells) were collected by centrifugation and resuspended in GR assay buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.5). Cells were lysed by 4 x 30 s beadbeating cycles. Twenty microliters of lysed cell supernatant was mixed with 100 µL GR assay buffer and 20 µL oxidized glutathione. The reactions were initiated with 50 µL NADPH, and oxidation of NADPH was measured kinetically over 10 min at 340 nm at an assay temperature of 25° C. Activity was calculated by $\Delta A_{340}min^{-1}mg^{-1}$ protein using an NADPH extinction coefficient of 0.00373 µM^{-1}.

APX activity was measured according to (Venisse, Gullner, & Brisset, 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 hydrogen peroxide. Oxidation of ascorbate was monitored spectrophotometrically as a decrease A_{290} (extinction coefficient 0.00168 µM^{-1}) over 10 min to determine APX activity (Venisse et al., 2001).

For ascorbate quantitation, 25 mL of culture (6 to 7 x 10^7 total cells) was pelleted and washed once in HPLC grade H$_2$O. Pellets were extracted in 2 mM EDTA containing 5 mM dithiothreitol and 1% orthophosphoric acid. Cells were broken using a bead beater (2 x 30s cycles) and samples were centrifuged for 30 min at 19,000 x g. Total cellular ascorbate levels (ascorbate + dehydroascorbate) were determined using a commercial kit (Ascorbate Assay Kit, Cayman Chemical. #700420).

The genome and transcriptome of UWO 241 were sequenced and assembled as described before (Cvetkovska et al., 2019). These datasets were screened for the presence of the genes encoding for enzymes of the AsA-GSH cycle. Previously identified genes from the model alga *Chlamydomonas reinhardtii* were obtained from the Phytozome database (v12, Joint Genome Institute) and used as a query. Genomic sequences with a high degree of identity (E-value cutoff 10^{-20}) were obtained and annotated using Geneious Prime (Biomatters Ltd, Auckland, New Zealand).

The amino acid sequence was predicted based on the gene coding sequence, and the identity of the enzyme was confirmed based on conserved motifs (Pitsch Witsch, & Baier, 2010; Wu and Wang, 2019). Multiple sequence alignments were performed using Clustal Omega (Sievers and Higgins, 2018), and protein localization was predicted using PSORT (Horton et al., 2007) and PredAlgo (Tardif et al., 2012). Sequence data for the UWO241 genes can be found in GenBank/EMBL database under accession numbers listed in Table S2.

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Photoinhibition assays

Short-term photoinhibitory and recovery treatments were performed on UWO 241 and SAG 49.72 cells grown under control growth conditions according to Pocock et al. (2007). Algal cells were harvested during mid-log phase of growth and placed under either high light (HL, 300 µmol m^{-2} s^{-1}) or low temperature (LT, 2°C) stress for 1 hr. For the HL treatment, cultures were maintained at their growth temperatures of 8°C (UWO 241) and 20°C (SAG 49.72). 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. Following the photoinhibitory treatment, cells were allowed to recover in the light (20 µmol m^{-2} s^{-1}) for 2 hr. Photoinhibition and recovery was monitored at regular time intervals as the decline and recovery in F_V/F_M, respectively.

Semi-quantitation of superoxide

Superoxide (O_2^-) levels were quantified according to (Förster, Osmond, & Pogson, 2005) with some modifications. A volume representing ~12,500 cells of UWO 241 or SAG 49.72 mid-log phase cultures grown under control conditions was treated with 20 µL of 1 mM nitroblue tetrazolium (NBT; Sigma) in the dark for 5 min prior to the stress treatment. Samples were then filtered onto 25 mm GF/C filters (Whatman, Cat No. 1822-025) and exposed to short-term stress of either LT (5° C) or HL (300 µmol m^{-2} s^{-1}) in an AlgaeTron 130 growth incubator (Photon Systems Instruments, Czech Republic) for up to 1 hr. Following treatment, filters were immediately immersed in 80% acetone to remove Chl, and then allowed to dry in a fume hood prior to imaging. O_2^- levels were measured semi-quantitatively by densitometric analyses using the program ImageJ (http://imagej.nih.gov/ij/).

Statistical Analyses

Statistical significance was determined using Student's paired t-test, uneven variance (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.

RESULTS

Growth physiology and PSII photochemistry

To compare the mechanisms by which the psychrophilic UWO 241 and the mesophilic SAG 49.72 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) (Table 1). First, it was confirmed that both strains could fully acclimate to each stress conditions by exhibiting log-phase growth and high photochemical activity in mid-log phase cultures. UWO 241 and SAG 49.72 acclimated to all long-term stress conditions as reflected in their ability to exhibit exponential growth and achieve high PSII photochemical efficiency (F_V/F_M) under stress conditions (Figure S1; Table 2). Despite the fact that UWO 241 tolerates significantly lower temperature and high salinity levels compared with SAG 49.72, the two strains generally exhibited comparable growth rates under control vs. stress conditions (Table 2). On the other hand, Chl a/b ratios were significantly lower in UWO 241 vs. SAG 49.72 across all growth conditions (Table 2). Moreover, SAG 49.72 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 (Table 2).

Both organisms exhibited qL values > 0.70 under control vs. stress conditions, with the exception of HL-cultures which exhibited lower qL in both species relative to controls (Table 2). Both strains exhibited a decrease in FPSII in response to HL which was accompanied by an increase in FNO and FNPQ in HL-SAG 49.72 and HL-UWO 241, respectively (Figure 1a,b). Last, we measured the capacity for NPQ under a range of measuring irradiance levels. The mesophilic strain maintained low NPQ levels over the range of irradiance levels regardless of whether cells were acclimated to control or long-term stress (Figure 2). In contrast, control UWO 241 cells exhibited significantly higher NPQ compared with that of SAG 49.72. Furthermore,

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acclimation to all stress conditions further increased UWO 241 NPQ capacity, with HL- and HS-UWO 241 exhibiting the highest NPQ values (Figure 2).

77K Chl a fluorescence emission spectra

77K emission spectra of whole cells of the mesophilic SAG 49.72 exhibited prominent fluorescence peaks at 684 nm and 714-716 nm consistent with LHCII-PSII and PSI fluorescence emission, respectively (Figure 3a). In contrast with the mesophile, UWO 241 exhibited major Chl a fluorescence emission peaks at 685 nm and 697 nm, but lacked a prominent emission peak for PSI at longer wavelengths 715-720 nm (Figure 3b). Acclimation to long-term stress resulted in changes in the 77K Chl a fluorescence emission spectra of SAG 49.72 (Figure 3a). Gaussian analysis of the fluorescence spectra revealed that SAG 49.72 exhibited a 1.5- to 3.2-fold decrease in the ratio of PSII/PSI fluorescence in response to LT, HL or HS stress (Table S1). In contrast, UWO 241 cells exhibited minimal changes in PSI fluorescence (Figure 3b), and only HS stress resulted in a minor decrease (1.18-fold relative to control) in the PSII/PSI ratio of UWO 241 (Table S1).

Photosystem I energy partitioning and activity

At the level of PSI energy partitioning, both organisms exhibited relatively high ΦPSI under all growth conditions, with the exception of HL which resulted a decrease in ΦPSI of 2.2 and 1.2-fold relative to controls in HL-SAG 49.72 and HL-UWO 241, respectively (Figure 1c,d). Both strains exhibited a concomitant increase in donor side limitation (ΦND) in response to HL; however, ΦND was higher in HL-SAG 49.72 relative to HL-UWO 241. Last, acclimation to all stress conditions resulted in lower acceptor side limitation (ΦNA) relative to control conditions in UWO 241 (Figure 1d).

PSI activity was monitored in mid-log cultures of both strains acclimated to control or long-term stress conditions by far red (FR) light inducible P700 photooxidation (Figure 4). The rise in absorbance at 820 nm (ΔA_{820}) is a relative measure of the fraction of photooxidizable P700 reaction centers, while rates of P700 re-reduction in the dark ($t_{\frac{1}{2}}{}^{red}$) reflect electron donation from alternative donors and mainly CEF (Ivanov et al., 1998). UWO 241 exhibited a significantly lower FR-inducible ΔA_{820} compared to SAG 49.72. Furthermore, ΔA_{820} values were below detection limits in HL-UWO 241 and HS-UWO 241 (Figure 4a). Last, UWO 241 cells grown in control and LT conditions exhibited significantly faster $t_{\frac{1}{2}}{}^{red}$ compared with SAG 49.72 grown under all conditions, with the exception of $t_{\frac{1}{2}}{}^{red}$ from HS-SAG 49.72 (Figure 4b).

Since we were unable to detect an FR-induced A_{820} signal for either HL-UWO 241 or HS-UWO 241, we performed the same measurement in the presence of red actinic light (AL) after treating the cells with DCMU to block electron transfer from PSII to PSI. Under AL, we were able to measure A_{820} in all UWO 241 cultures (Figure 5). In agreement with the FR measurements of P_{700} photooxidation, acclimation to all three long-term stress conditions caused a significant decrease in ΔA_{820} relative to the UWO 241 cells grown in control conditions (Figure 5a). Moreover, following AL excitation, $t_{\frac{1}{2}}{}^{red}$ was 1.3 to 1.6-fold faster under all stress conditions relative to that of control conditions (Figure 5b, c). These results agreed with a recent report which compared P700 photo-oxidation with electrochromic shift measurements to show that UWO 241 possesses increased CEF under high salt conditions (Karla et al. 2020).

Relationship between NPQ and CEF

We determined whether high CEF was associated with a stronger capacity for NPQ (Figure 6). All UWO 241 cultures exhibited faster rates of P700$^+$ re-reduction and higher maximum NPQ levels relative to SAG 49.72. A strong negative correlation was observed between NPQ capacity and P700$^+$ re-reduction rate within either the dataset across all experiments or within UWO 241 samples ($r^2 = 0.93$ and 0.99, respectively). In contrast, SAG 49.72 exhibited a weak positive correlation between NPQ and P700$^+$ re-reduction rate ($r^2 = 0.52$).

AsA-GSH pathway

The enzymes APX and GR are key enzymes of an ROS detoxification pathway, the AsA-GSH cycle, catalyzing the first step of the pathway and regeneration of reduced glutathione, respectively (Noctor & Foyer, 1998).

Enzymatic assays revealed low activity for either APX or GR in SAG 49.72, grown under control or HL, LT and HS stress conditions (Figure 7A). In contrast, UWO 241 exhibited significantly higher activity for both enzymes under control and all stress treatments relative to SAG 49.72 (Figure. 7). GR activity was highest in HS-UWO 241 cells, while APX activity was highest in LT-UWO 241 cells relative to controls. Last, AsA-GSH pathway substrate, ascorbate, was significantly higher in UWO 241 compared with values typically reported for other algae (Gest, Gautier, & Stevens, 2013). Total cellular ascorbate 9.61 ± 1.20 and 18.52 ± 1.90 mM ascorbate under control and high salinity conditions, respectively, compared with $0.93 \pm$ in the mesophile, *C. reinhardtii* (Figure S2).

Screening of the UWO 241 genome and transcriptome revealed homologs for all genes involved in the AsA-GSH cycle, with the exception of monodehydroascorbate reductase (MDHAR) (Tables S2 and S3). The genome of UWO 241 encodes 5 genes identified as APX (APX1, APX2-A to –D), which share a high sequence similarity with homologous genes from other photosynthetic organisms and the presence of conserved motifs involved in APX catalytic function (Figure S3a). Four of these genes (APX2-A to –D) are found on the same contig in a head-to-tail orientation and share a high sequence similarity (83.1-93.3%), suggesting a recent gene duplication event (Figure S3b). This is in contrast with other green algae that typically encode one or two APX genes with confirmed APX activity (Pitsch et al., 2010; Gest et al., 2013). All other genes, including GR, were present as a single copy and shared a high sequence identity with homologous genes from *C. reinhardtii* (Table S3).

Short-term photoinhibitory assays

Given the differential responses to long-term stress between the psychrophile and mesophile, we compared sensitivity and recovery capacity of UWO 241 and SAG 49.72 to low temperature- and high light-induced photoinhibition by monitoring changes in the PSII photochemistry parameter, F_V/F_M. First, we tested sensitivity of both organisms to low temperature versus high light at variable incubation temperatures (2°C, 8°C, 20°C) and irradiances (50 µmol m^{-2} s^{-1}, 300 µmol m^{-2} s^{-1}, 1000 µmol m^{-2} s^{-1}). Decline in F_V/F_M was recorded at various time points over 3 h (Figure S4). At 8°C and 20°C incubation temperatures, UWO 241 exhibited higher sensitivity to both medium and high light treatments, relative to SAG 49.72. However, when the assay temperature was decreased to 2°C, SAG 49.72 exhibited higher photoinhibition under low light, while both strains exhibited comparable sensitivity to medium or high light treatment (Figure S4). Thus, for the recovery assays, we choose to test samples for 1 hr under two photoinhibitory treatments: i) low temperature (LT, 2°C) and ii) high light (HL, 300 µmol m^{-2} s^{-1}). In agreement with our results from the preliminary assays, UWO 241 and SAG 49.72 exhibited differential sensitivity to HL and LT treatments, respectively. 1 hr of HL-treatment resulted in 62% decrease in F_V/F_M in UWO 241, while LT caused a 32% decrease in F_V/F_M in SAG 49.72 (Figure 8a). Conversely, LT and HL treatment cause only a moderate (<10%) decrease in F_V/F_M in UWO 241 and SAG 49.72, respectively. In addition, HL-treatment of UWO 241 resulted in almost a complete loss of qL which was accompanied by an increase in FNPQ from 0.07 to 0.47. LT-treated SAG 49.72 exhibited only a 67% reduction in qL. Both strains exhibited comparable increases in FNPQ in response to the LT treatment (Figure 8b).

Despite the strain differences in sensitivity to short-term photooxidative stress, both organisms exhibited full recovery to initial F_V/F_M levels within 2 h (Figure 8a). The recovery was most dramatic in HL-treated UWO 241 which exhibited a rapid rate of recovery within the first 15 mins (Figure 8a). Strain- and treatment-specific differences were observed at the level of recovery of qL and FNPQ. Surprisingly, despite restoration of F_V/F_M to pre-treatment values, HL-treated cells of UWO 241 were unable to restore qL to initial levels: qL remained close to zero even after 2 hours of recovery (Figure 8b). Similarly, FNPQ did not relax in HL-treated UWO 241 during the recovery phase (Figure 8c). In contrast, both strains exhibited similar trends in relaxation of FNPQ following LT treatment (Figure 8c).

Last, we monitored whether short-term HL- or LT-treatments were accompanied by accumulation of an ROS, using the dye NBT as a semi-quantitative measurement of O_2^- (Figure 9). The mesophile SAG 49.72 exhibited significantly higher levels of O_2^- relative to pre-treated cells after 1 hr of short-term HL or LT treatment. Conversely, UWO 241 exhibited no significant change in ROS levels after the short-term stress

treatments (Figure 9).

DISCUSSION

This study examined whether two *Chlamydomonas* species adapted to extreme contrasts in their native environments rely upon comparable strategies for survival under long-term stress. SAG 49.72 was originally isolated from a temperate lake: it's a mesophilic species and possesses limited ability to acclimate to either salinity or low temperature stress (Pocock et al., 2011; Szyszka et al., 2007). In stark contrast, within the deep photic zone of Lake Bonney, Antarctica, UWO 241 has survived under permanent low temperature and hypersalinity stress for at least 1000 years, based on estimates of the last occurrence of ice-free conditions (Morgan-Kiss et al., 2006). Our results confirmed that although both the mesophilic SAG 49.72 and the psychrophilic UWO 241 exhibited the ability to grow robustly under high light, low temperature or high salinity, their tolerance levels and long-term acclimatory strategies to these environmental stressors were markedly different. For the mesophilic SAG 49.72, long-term acclimation could be summarized into maintenance of photostasis by adjustments in PSII antenna size and PSII-PSI energy distribution, both classic long-term acclimatory mechanisms described for other model algal species (Maxwell, Falk, Trick, & Hüner, 1994; Tanaka & Melis, 1997). In contrast, the psychrophilic UWO 241 relies upon constitutive CEF and continuous ROS detoxification capacity to provide photoprotection to both PSII and PSI under long-term stress.

Long-term stress acclimation in SAG 49.72 involved an increase in the ratio of Chl a/b and a concomitant decrease in PSII/PSI at the level of 77K Chl a fluorescence emission. Higher Chl a/b ratios in response to long-term stress have been reported across many algae and plants and coincides with a decrease in the size of LHCII (Maxwell et al., 1994; Smith et al., 1990; Wilson & Hüner, 2000). Decreases in PSII/PSI stoichiometry under either high light or low temperature stress are also well documented and reflects enhanced distribution of absorbed light energy in favor of PSI (Smith et al., 1990; Velitchkova, Popova, Faik, Gerganova, & Ivanov, 2020). On the other hand, UWO 241 maintained very low Chl a/b ratios across all treatments, indicating that it does not need to adjust LHCII antenna size even when exposed to either growth temperatures approaching 0°C or hypersalinity. These results support previous observations that UWO 241 maintains a relatively large oligomeric LHCII (Morgan et al. 1998). Szyszka et al. (2007) also observed that UWO 241 does not modulate abundance of two major LHCII polypeptides in response to variable light intensity. Morgan-Kiss et al. (2002) demonstrated that UWO 241 is also unable to undergo state transitions. More recently, Szyszka-Mroz and colleagues reported that the psychrophile relies instead on a poorly understood constitutive spill-over mechanism under HS growth conditions (Szyszka-Mroz et al., 2019). Thus, UWO 241 is a natural variant lacking state transitions that maintains a relatively large LHCII and high PSII relative to PSI content under long-term stress. Despite an apparent lack of some classic acclimatory mechanisms, stress-acclimated cells of UWO 241 maintained a high qL and comparable energy partitioning relative to control conditions, suggesting that the psychrophile deals with high excitation pressure other strategies.

CEF is an essential process in plants and algae for balancing ATP/NADPH and photoprotection; although, most studies have been restricted to considering CEF during short-term stress. Early reports identified that UWO 241 exhibits relatively high rates of PSI-driven CEF compared with mesophilic strains (Morgan-Kiss et al., 2002b; Morgan-Kiss et al., 2006). Maximal CEF requires restructuring of the photosynthetic apparatus and assembly of a novel PSI supercomplex (Kalra et al., 2020; Szyszka-Mroz et al., 2015). The UWO 241 supercomplex is distinct from that of previously described complex from *C. reinhardtii* (Iwai et al., 2010) because the former is not associated with state-transition-inducing treatments and it lacks typical PSI 77K fluorescence emission despite the presence of many PSI core proteins (Kalra et al., 2020; Szyszka-Mroz et al., 2015). Here we show that UWO 241 exhibits faster CEF rates under salinity stress, high light or low temperatures, suggesting that the extremophile relies on sustained CEF as a general long-term acclimatory strategy.

CEF generates additional transthylakoid proton motive force which is used for several purposes, including balancing ATP/NADPH production and photoprotection of both PSII and PSI (Bulte, Gans, Febeille, & Wollman, 1990; Chaux, Peltier, & Johnson, 2015; He et al., 2015; Lucker & Kramer, 2013; Yamori et al.,

2016). Kalra and colleagues showed that under long-term HS stress CEF serves multiple purposes in UWO 241, including additional ATP production as well as constitutive photoprotection (Kalra et al., 2020). Higher ATP levels are used in part to support enhanced CBB pathway activity which supplies substrates for storage compounds (starch), osmoregulants (glycerol), as well as the shikimate pathway (Kalra et al., 2020). It is likely that CEF is utilized for similar processes when UWO 241 is acclimated to HL or LT. This current study provides evidence that high CEF in all three stress conditions is associated with enhanced photoprotection of PSII. In UWO 241, CEF rates exhibited a strong correlation with higher capacity for NPQ compared. In contrast, SAG 49.72 had low CEF under all conditions and no correlation between NPQ and CEF (Figure 6). This suggests constitutive capacity for PSII protection in the psychrophile owing to enhanced CEF-generated pmf.

High CEF also provides PSI photoprotection. UWO 241 cultures acclimated to all three long-term stress conditions were associated with reduced levels of FNA relative to control-grown UWO 241 cells. These data suggest that CEF also contributes to protection of PSI by preventing accumulation of reduced Fd and minimizing acceptor side limitation. Over-reduction of PSI manifests as production of the ROS, O_2^- (Asada, 1999). We show that UWO 241 possesses remarkable ability to avoid O_2^- accumulation: cells exposed to either short-term LT or HL stress exhibited minimal accumulation of this ROS. This ability to keep O_2^- levels lows is likely due to CEF-associated prevention of PSI acceptor side limitation. In contrast the mesophile exhibited significant levels of O_2^- when exposed to the same conditions. While PSII is typically considered sensitive to all environmental stresses, PSI photodamage occurs under specific environmental conditions, including drought, high salinity and low temperature, and repair of PSI is slow and inefficient (Huang, Yang, Zhang, Zhang, & Cao, 2012; Huang, Yang, Hu, & Zhang, 2016; Huang, Zhang, Xu, & Liu, 2017; A. G. Ivanov et al., 1998; Yamori et al., 2016; Zhang & Scheller, 2004). Similarly, elevated CEF protects PSI from low temperature-associated photoinhibition during the winter-spring transition in Scots pine (Yang et al. 2020), which fits well with an earlier hypothesis that UWO 241 is an 'evergreen alga' during the polar night (Morgan-Kiss et al. 2006). Thus, PSI photoinhibition is a deleterious consequence for survival under long-term stress. We suggest that constitutive CEF serves multiple roles, simultaneously plays critical roles in protecting both PSII and PSI from photo-damage in UWO 241 for survival under long-term environmental stress.

UUWO 241 exhibits constitutive protection of PSII and PSI in UWO 241 by minimizing ROS production; however, there is also evidence that the psychrophile possesses enhanced ability for ROS detoxification. The AsA-GSH pathway is a major ROS detoxification pathway in plants and is responsible for regeneration of the antioxidant ascorbate (Foyer & Noctor, 2012; Foyer & Shigeoka, 2011). The AsA-GSH pathway involves four enzymes, ascorbate peroxidase (APX), monohydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor & Foyer, 1998). Plants express multiple isoforms of each enzyme, in particular APX (Pitsch et al. 2010; Teixeira, Menezes-Benavente, Margis, & Margis-Pinheiro, 2004). High concentrations of ascorbate accumulate in plants, particularly under stress conditions, including high light, low temperatures and high salinity (Bartoli, Buet, Grozeff, Galatro, & Simontacchi, 2017; Maruta & Ishikawa, 2017; Wildi & Lütz, 1996; Zechmann, Stumpe, & Mauch, 2011; Zhang et al., 2011). On the other hand, cyanobacteria and algae exhibit significantly lower levels of ascorbate and possess only one isoform or are missing one or more enzymes of the AsA-GSH pathway (Gest, Gautier, & Stevens, 2013). For example, the model *C. reinhardtii* appears to lack the thylakoid-bound APX found in plants, expressing only a single isoform of APX which is localized to the stroma (Pitsch et al., 2010). A second APX2 isoform has been predicted to localize to the chloroplast, but its function has not been studied (Wu and Wang, 2019). Three pieces of evidence indicate that UWO 241 may rely on the AsA-GSH pathway to a greater extent than previously appreciated in other algal species. First, activity of two enzymes, APX and GR, are constitutively high in UWO 241 relative to the mesophile SAG 49.72 under both control and all long-term stress conditions. Second, UWO 241 cells accumulated millimolar levels of the substrate ascorbate. Last, unlike other algae studied thus far, UWO 241 appears to possess more isoforms of several enzymes necessary for ascorbate cycling. A search of a previously published transcriptome of UWO 241 (Raymond & Morgan-Kiss, 2013) revealed multiple potential homologues for enzymes of the AsA pathway, including 3 APX, 3 DHAR, and 3

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GR genes (Table S2). These genes were also detected at the level of the genome, with four APX genes present as highly similar tandem duplicates (Table S3; Figure S3). In addition, one of the putative UWO 241 APX proteins is related to a plant thylakoid-bound isoform from *Triticum aestivum* . APX catalyzes the oxidation of ascorbate by H_2O_2, while DHAR and GR work in concert to regenerate glutathione. Similarly, Zhang and colleagues (Zhang et al. 2020) recently reported that the Antarctica sea ice alga, *Chlamydomonas* sp. ICE-L, exhibited gene expansion of the APX enzyme and increased APX activity. We did not identify an isoform for MDHAR in the genome or transcriptome. This enzyme is prevalent in plant genomes, but often missing from algae which can also regenerate Asc by non-enzymatic dismutation of MDHA to (Gest, Gautier, & Stevens, 2013). The additional isoforms may be localized to different cellular compartments, as in plants, or may contribute to constitutively high AsA-GSH pathway activity. Gene duplications have been shown for several other UWO 241 genes including photosynthetic ferredoxin (Cvetkovska et al., 2018), chlorophyllide a oxygenase (Cvetkovska et al., 2019) and the chloroplast kinase Stl-1 (Szyszka-Mroz et al., 2019). Massive expansion of multiple gene families involved in photoprotection, DNA repair, ROS detoxification, and several other essential processes for environmental adaptation, were also detected in the genome of *C.* sp. ICE-L (Zhang et al. 2020).

Conclusions and renaming of UWO 241

This study further expands more than two decades of work on the enigmatic, Antarctic alga, *Chlamydomonas* sp. UWO 241 which have documented novel adaptation strategies to survive permanent extreme conditions. The taxonomic identity of UWO 241 has been the subject of debate: originally identified on a morphological basis as *C. subcaudata* by Priscu & Neale (1995), the organism was erroneously identified as *C. raudensis* UWO 241 (Pocock et al., 2004). Recently, a thorough revisiting on the taxonomy of the strain performed by Possmayer et al. (Possmayer et al., 2016) concluded that UWO 241 represents a unique lineage within the Moewusinia clade, and it was therefore renamed *Chlamydomonas* sp. UWO 241 as a place holder. Molecular phylogenetic analysis of the full length 18S rRNA gene revealed that the closest known relative of UWO 241 is a marine alga, *Chlamydomonas parkeae* SAG 24.89 (95% identity). Given the geographical isolation and unique physiology, combined with the molecular and genomic analyses, we suggest that UWO 241 is a unique strain. According to requirements of the International Code of Nomenclature for algae, fungi, and plants (McNeill et al., 2012) we propose to rename the strain *Chlamydomonas priscuii* in recognition of John C. Priscu, the investigator who originally isolated the strain in 1995 (Neale & Priscu, 1995).

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FIGURE LEGENDS

FIGURE 1. Effect of long-term stress acclimation on partitioning of PSII (a) and PSI (b) in UWO 241 and SAG 49.72 grown under control and long term stress conditions. ΦNO, non-regulated energy dissipation; ΦNPQ, non-photochemical energy quenching; ΦPSII, PSII quantum yield; ΦPSI, PSI quantum yield; ΦND, donor side limitation; ΦNA, acceptor side limitation. C, Control; HL, High Light; LT, Low Temperature; HS, High Salt. Values are means with standard deviations (n=3). All parameters were measured at growth irradiance (see Table 1).

FIGURE 2. Capacity for nonphotochemical quenching in UWO 241 (UWO) and SAG 49.72 (SAG) grown under control and high stress conditions. C, Control; HL, high light; LT, low temperature; HS, high salt.

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Letters - a, statistical significance between control vs. stress within one algal species; b, statistical significance between UWO 241 vs. SAG 49.72 when grown under same treatment (n=3; p<0.05).

FIGURE 3. 77K Chl a fluorescence emission spectra of the mesophile SAG 49.72 (a) and the psychrophile UWO 241 (b) and acclimated to control and long-term stress treatments. C, Control; HL, high light; LT, low temperature; HS, high salt.

FIGURE 4. Photosystem I (P700) oxidation/reduction of the mesophile SAG 49.72 and the psychrophile UWO 241 grown under control and long-term stress conditions. a. Oxidation state of P_{700}. b. Re-reduction kinetics of $P_{700}+$. P_{700} oxidation/reduction was monitored in the presence of far red light. Letters - a, statistical significance between control vs. stress within one algal species; b, statistical significance between UWO 241 vs. SAG 49.72 when grown under same treatment (n=3; p<0.05). C, Control; HL, high light; LT, low temperature; HS, high salt; ND, not detectable.

FIGURE 5. Photosystem I oxidation/reduction kinetics in UWO 241 grown under control and long-term stress conditions. P_{700} oxidation/reduction was monitored in the presence of red actinic light following DCMU pretreatment. (a) Oxidation state of P_{700}. (b) Re-reduction kinetics of $P_{700}+$. Letters statistical significance between control vs. stress (n=3; p<0.05). C, Control; HL, high light; LT, low temperature; HS, high salt. (c) P700+ re-reduction kinetics.

FIGURE 6 . NPQ and CEF exhibit a linear relationship. C. raudensis SAG 49.72 and C. sp. UWO241 grown under control and long-term stress conditions. NPQ axis represents maximum NPQ values determined from light response curves. CEF represents P700 re-reduction rates. Dashed and solid lines show linear regression for all data points or individual organisms, respectively (n=3 or 7-9 for NPQ and CEF, respectively).

FIGURE 7. Activity of AsA-GSH pathway enzymes, ascorbate peroxidase (APX, a) and glutathione reductase (GR, b) the mesophile SAG 49.72 and the psychrophile UWO 241 grown under control and long-term stress conditions. Letters - a, statistical significance between control vs. stress within one algal species; b, statistical significance between UWO 241 vs. SAG 49.72 when grown under same treatment (n=3; p<0.05). C, Control; HL= high light; LT, low temperature; HS, high salt.

FIGURE 8. Photoinhibition and recovery in the mesophile SAG 49.72 and the psychrophile UWO 241. Cultures grown under control conditions exposed to either high light (HL, 300 μmol m^{-2}s^{-1}) or low temperature LT, (2°C) short-term stress for 60 min then returned to control conditions for 120 min to recover. (a) Percent photoinhibition was determined by loss of photochemical ability (F_V/F_M) relative to time zero. (b) Photochemical quenching, qL. C. nonphotochemical quenching, FNPQ (n=3).

FIGURE 9. Production of reactive oxygen species in UWO 241 (P) vs. SAG 49.72 (M) 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 in the presence of NBT dye to detect superoxide. Data is normalized to time 0. Letters - a, statistical significance between control vs. stress within one algal species; b, statistical significance between UWO 241 vs. SAG 49.72 when grown under same treatment (n=3; p<0.05).

Table 1. Growth conditions for UWO 241 and SAG 49.72 used in this study.

Growth Condition	UWO 241	SAG 49.72
Control (C)	8°C 0.43 mM NaCl 50 μmol m^{-2} s^{-1}	20°C 0.43 mM NaCl 50 μmol m^{-2} s^{-1}
High light (HL)	250 μmol m^{-2} s^{-1}	500 μmol m^{-2} s^{-1}
Low temperature (LT)	2 °C	11 °C
High Salinity (HS)	700 mM NaCl	100 mM NaCl

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Growth Condition	Max Growth Rate (h^{-1})	F_V/F_M	qL	Total Chlorophyll (µg ml^{-1})	Chl a/b (g:g)
UWO 241	UWO 241	UWO 241			
C	0.031 ± 0.002	0.67 ± 0.02^b	0.72 ± 0.08^b	6.99 ± 1.09	1.00 ± 0.02^b
HL	0.034 ± 0.001	0.60 ± 0.01^a	0.60 ± 0.05^b	$4.02 \pm 0.45^{a,b}$	0.80 ± 0.14^b
LT	$0.018 \pm 0.002^{a,b}$	$0.55 \pm 0.02^{a,b}$	0.82 ± 0.04^b	4.79 ± 0.59^a	0.97 ± 0.10^b
HS	0.034 ± 0.002^b	0.60 ± 0.05^b	0.70 ± 0.10^b	5.06 ± 0.46	0.90 ± 0.10^b
SAG 49.72	SAG 49.72	SAG 49.72			
C	0.030 ± 0.001	0.70 ± 0.02	0.94 ± 0.04	9.26 ± 1.62	2.28 ± 0.77
HL	0.087 ± 0.002^a	0.65 ± 0.04	0.24 ± 0.00^a	6.86 ± 0.08	4.29 ± 0.29
LT	0.024 ± 0.001^a	0.66 ± 0.01^a	1.00 ± 0.00	4.31 ± 0.15^a	5.66 ± 0.29^a
HS	0.016 ± 0.002^a	0.66 ± 0.04	0.94 ± 0.09	8.13 ± 0.84	3.51 ± 0.05

Table 2. Growth physiology parameters in cultures of UWO 241 and SAG 49.72 grown under control versus long-term stress conditions. Values are means with standard deviations (n=3 biological replicates). Superscript letters - [a], statistical significance between control vs. stress within one algal species;[b], statistical significance between UWO 241 vs. SAG 49.72 when grown under same treatment (p<0.05). F_V/F_M, maximum photochemical efficiency; qL, photochemical quenching, measured at growth irradiance (see Table 1).

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(a)

P700 Oxidation state (ΔA 820)

C   HL   LT   HS

(b)

OEF (P700) re-oxidation rate (ms)

C   HL   LT   HS

(c)

P700 Reduction (ΔA820)

C
HS
LT
HL

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