LARGE-SCALE BIOLOGY ARTICLE

Systems-Wide Analysis of Acclimation Responses to Long-Term Heat Stress and Recovery in the Photosynthetic Model Organism Chlamydomonas reinhardtii

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We applied a top-down systems biology approach to understand how Chlamydomonas reinhardtii acclimates to long-term heat stress (HS) and recovers from it. For this, we shifted cells from 25 to 42°C for 24 h and back to 25°C for ≥8 h and monitored abundances of 1856 proteins/protein groups, 99 polar and 185 lipophilic metabolites, and cytological and photosynthesis parameters. Our data indicate that acclimation of Chlamydomonas to long-term HS consists of a temporally ordered, orchestrated implementation of response elements at various system levels. These comprise (1) cell cycle arrest; (2) catabolism of larger molecules to generate compounds with roles in stress protection; (3) accumulation of molecular chaperones to restore protein homeostasis together with compatible solutes; (4) redirection of photosynthetic energy and reducing power from the Calvin cycle to the de novo synthesis of saturated fatty acids to replace polyunsaturated ones in membrane lipids, which are deposited in lipid bodies; and (5) when sinks for photosynthetic energy and reducing power are depleted, resumption of Calvin cycle activity associated with increased photorespiration, accumulation of reactive oxygen species scavengers, and throttling of linear electron flow by antenna uncoupling. During recovery from HS, cells appear to focus on processes allowing rapid resumption of growth rather than restoring pre-HS conditions.

INTRODUCTION

Global warming will increase occurrences of extreme heat stress (HS). In particular when occurring during anthesis, HS may severely reduce crop yield and therefore is predicted to pose a significant problem for agriculture (Lobell et al., 2011; Deryng et al., 2014). To identify entry points for metabolic engineering or chemical approaches aiming to improve crop plant resistance to HS, a comprehensive understanding of how plants respond to HS and recover from it is required. Although many aspects of how plant systems respond to HS have been studied in the past, many questions remain open (Sharkey, 2005; Mittler et al., 2012).

One of them is how plants sense heat and integrate signals from sensors to elicit a coordinated response. A universally conserved trigger for HS responses is the accumulation of unfolded proteins. In eukaryotes, these are known to be sensed in the cytosol, the endoplasmic reticulum (ER), and mitochondria and signaled to the nucleus to initiate appropriate transcriptional responses (Voellmy and Boellmann, 2007; Walter and Ron, 2011; Haynes et al., 2013). Recent work suggested that an unfolded protein response also exists in the chloroplast (Yu et al., 2012; Schmollinger et al., 2013; Ramundo et al., 2014). Plasma membrane fluidity has been identified as an important temperature sensor in land plants that appears to lie upstream of the unfolded protein response (Sangwan et al., 2002; Suri and Dhindsa, 2008; Saidi et al., 2009; Wu et al., 2012). Increased membrane fluidity appears to open calcium channels in the plasma membrane and inflowing calcium to trigger signaling cascades, including an H₂O₂ burst (Königshofer et al., 2008), leading to the activation of the HS response. HS also was shown to result in the activation of phospholipase D and phosphatidylinositol-4-phosphate 5-kinase, thus leading to the accumulation of lipid signaling molecules that might trigger specific HS responses (Mishkind et al., 2009). Yet unidentified triggers may stem from metabolism, with metabolites serving in HS signaling that change in abundance due to temperature-induced changes of enzyme activities.
Another open question is related to how and why HS affects photosynthetic activity. Photosystem II (PSII) is considered the most thermosensitive complex of the photosynthetic light reactions (Berry and Bjorkman, 1980). However, the temperatures at which PSII damage was observed often were not physiological and the reduced rates of photosynthesis at moderate HS are more likely to be caused by reduced capacities of downstream reactions than by PSII damage (Sharkey, 2005; Zhang and Sharkey, 2009; Sharkey and Zhang, 2010). One of the downstream pathways affected by moderate HS is the Calvin cycle (Weis, 1981), and a key activity affected is that of Rubisco activase (Feller et al., 1998). Rubisco activase catalyzes the release from the Rubisco active site of ribulose-1,5-bisphosphate and other sugar phosphates that, when bound to Rubisco prior to its carbamylation and Mg\(^{2+}\) binding, inactivate the enzyme (Jensen, 2000). The question is whether the inactivation of thermolabile Rubisco activase by HS is an undesired effect, or rather a mechanism implemented specifically as part of a regulated response to moderate HS, as suggested previously (Sharkey, 2005; Sage et al., 2008; Sharkey and Zhang, 2010).

Recently, we used a shotgun proteomics approach based on \(^{15}\)N metabolic labeling to monitor changes in abundances of the soluble proteome in heat stressed Chlamydomonas reinhardtii cells during a 3-h time course (Mühlhaus et al., 2011). When comparing results on Chlamydomonas with proteomics studies conducted on various land plants (Skylas et al., 2002; Majoul et al., 2003, 2004; Süle et al., 2004; Ferreira et al., 2006; Lee et al., 2007; Palmblad et al., 2008; Valcu et al., 2008; Xu and Huang, 2008; Scafaro et al., 2010; Xu and Huang, 2010), we concluded that the upregulation of chaperones, FKBP65 orthologs, and thiolamine biosynthesis protein and the downregulation of ferredoxin-NADP(H) oxidoreductase, Rubisco, methionine synthase, adenosylhomocysteinase, and S-adenosylmethionine synthetase are conserved in the green lineage. However, there were also many inconsistencies between these studies. An intriguing discrepancy was that in Chlamydomonas we could not detect any increase in the abundance of proteins involved in redox regulation/scavenging of reactive oxygen species, which was observed in several studies on land plants (Ferreira et al., 2006; Lee et al., 2007; Palmblad et al., 2008; Xu and Huang, 2008; Scafaro et al., 2010). Whether these inconsistencies were due to different durations of HS monitored, the different methodologies applied (2D-PAGE versus shotgun proteomics), or organism-specific responses remains unclear.

Top-down systems biology approaches, where responses at multiple system levels are monitored over time and integrated to a more holistic picture, appear helpful to shed light on the many open questions regarding responses of plant cells to HS. In fact, Chlamydomonas is an ideal plant model for such approaches because (1) as a single-celled organism all cells are of the same type and different cell cycle stages may be averaged out by growing cells in asynchronous cultures, (2) cells can be cultured under highly controlled conditions and changes in environmental conditions applied homogeneously, (3) gene families are less complex than those of land plants (Merchant et al., 2007). Accordingly, several recent studies have taken advantage of these characteristics to deepen our understanding of acclimation responses to N starvation (Blaby et al., 2013; Schmollinger et al., 2014), copper and iron limitation (Castruita et al., 2011; Höhner et al., 2013), anoxia (Hemschemeier et al., 2013), or increasing light (Mettler et al., 2014). In this study, we employed a top-down systems biology approach in which we monitored changes in the proteome (soluble and membrane proteins), metabolome, and lipidome as well as changes of many cytological and physiological parameters in Chlamydomonas during long-term HS and recovery. By integrating data from the different systems levels, we were able to obtain many insights that provide tentative answers to some of the open questions outlined and will be the groundwork for further experiments.

**RESULTS**

**Experimental Setup**

To monitor acclimation responses of Chlamydomonas to HS, we subjected cells growing under mixotrophic conditions in the exponential phase at 25°C to a 42°C HS treatment for 24 h and for recovery transferred cells back to 25°C for up to 24 h (Figure 1). Chlamydomonas cells were previously shown to fully recover from a 24-h HS treatment at 42°C (Mühlhaus et al., 2011). During this HS and recovery time course, we monitored changes in the proteome, metabolome, and lipidome, as well as cytological and physiological parameters. Differences in sampling frequency on the various system levels are based on the different time scales at which they change and on cost considerations.

**Cytological Parameters**

We first monitored several cytological parameters during the HS and recovery time course, i.e., cell numbers, cell size distribution, cellular ultrastructure, and contents of DNA, protein, chlorophyll, triacylglycerol (TAG), and starch. At 25°C, cells grew exponentially with a generation time of \(~8\) to 9 h (Figure 2A). Immediately after transfer to 42°C, cells stopped dividing and this division arrest lasted until \(~8\) h of recovery when cells resumed exponential growth. To test whether the cell division arrest was accompanied by an arrest of DNA replication, we determined the ploidy level of the cells during HS and recovery by flow cytometry (Figure 2B). In our study, we used cells grown asynchronously under mixotrophic conditions. Accordingly, before HS, 77% of the cells were 1n, 21.7% were 2n, while only \(~1.3\)% were 4n (note that vegetative Chlamydomonas cells are haploid; Harris, 2001). This distribution hardly changed during the 24 h HS treatment; consequently, the same was true for the average DNA content. DNA replication resumed with a short lag phase of 2 to 3 h after transferring cells back to 25°C. The fraction of 1n cells decreased, while that of 2n and 4n cells increased. After \(~7\) to 8 h of recovery, the distribution of 1n and 2n cells was equal and later 2n and 4n cells became even more abundant. A trend back to the initial distribution was observed after 24 h of recovery.

At 25°C, cells had a mean diameter of 4.9 \(\mu\) m, which increased to 6 \(\mu\) m during the 24 h HS treatment (Figure 2C). The increase in cell diameter was more pronounced during the first 7 h of HS than at later time points. Within the first 7 h of recovery, the average cell diameter continued to increase to a peak value of 6.4 \(\mu\) m and began to decline when cells started dividing again. Hence, while cell division was arrested during heat stress, cells continued to grow at a slow rate.
At 25°C, the average protein content per cell was 13.9 pg (Figure 2D). It increased by 57% to 21.8 pg during the 24 h HS treatment, continued to increase by 10% to a peak value of 24 pg during the first 8 h of recovery, and dropped to 20.1 pg after 24 h of recovery when cells were dividing again. The average chlorophyll content per cell was 0.84 pg. It increased by 24% to 1.04 pg during 24 h HS and declined only slightly during 24 h of recovery. Strikingly, after 24 h of recovery average cell diameter and average contents of DNA, protein and chlorophyll were higher than pre-HS values, although cells had divided ~1.6 times.

The cellular TAG content increased dramatically within the first 2 h of HS, but declined mildly between 2 and 24 h of HS (Figure 2E). Immediately after transfer to 25°C, TAG levels dropped slightly, but stayed constant during the remaining 8 h monitored in the recovery phase. In contrast to the other cellular macromolecules investigated, the starch content per cell declined within the first 2 h of HS by 28%, but during the remaining 22 h of HS increased to levels that were 46% above those present before HS. After transferring cells back to 25°C, the starch content rose strongly and after 8 h recovery was 2.7-fold higher than before the onset of HS.

Finally, electron microscopy images were taken to reveal structural rearrangements and possible cell damage during heat stress and recovery (Figure 2F). Based on the analysis of 520 images of cells from 0, 3, 12, and 24 h HS, and from 4 and 8 h recovery, cell damage occurred only sporadically. Consistent with the increase in cellular TAG content detected by liquid chromatography-mass spectrometry (LC-MS), we observed the accumulation of large lipid bodies during HS. Many of these lipid bodies were darkly stained and often contained less stained spots. Consistent with the HS-induced 46% increase in starch content determined by colorimetry, an increase in quantity and size of starch granules was also evident from the electron microscopy images (Figure 2F). Quantification of starch granules (excluding pyrenoid starch sheaths) by planimetry revealed an increase by ~18.5% (Supplemental Figure 1). No obvious changes in number, density, and stacking of thylakoid membranes were observed. However, after 12 h of HS, we observed the formation of aberrant structures at nodal points of several thylakoid membrane layers. These strongly resemble prolamellar body (PLB)-like structures previously described in *Chlamydomonas* mutants with reduced levels of the vesicle inducing protein in plastids (VIPP1), which was suggested to play a role in biogenesis/maintenance of thylakoid membrane protein complexes (Nordhues et al., 2012). PLB-like structures were not observed after 3 h HS and occurred in only 3% of the cells after 12 h HS (Supplemental Figure 2A). However, after 24 h HS, PLB-like structures were observed in 45% of the cells. This percentage stayed constant during the first 4 h of recovery and decreased to 27% after 8 h recovery. At this time only residual PLB-like structures were left (Figure 2F; Supplemental Figure 2B).

**Photosynthesis and Respiration**

To investigate effects of HS on the photosystem (PS) integrity and on efficiency of energy coupling of light-harvesting complexes (LHCs) to photosystem I (PSI) and PSII, we recorded 77K fluorescence spectra during the HS and recovery time course. As shown in Figure 3A, the ratio of the emission maxima for PSII (~687 nm) to PSI (~713 nm) increased strongly during the first 12 h of HS and recovered thereafter, but the pre-HS ratio was not regained after 8 h recovery. A similar pattern was observed for a “shoulder” occurring in the spectra at ~681 nm and a blue shift of the PSI emission peak from ~713.5 nm under nonstress conditions to ~710.5 nm after 24 h of HS (Figures 3B to 3E). These results are indicative of detachment of LHCII and LHCI from PSI and PSI, respectively (Armond et al., 1978; Naumann et al., 2005).

We further determined electron flow through PSII at different light intensities in cells harvested during the HS and recovery time course based on chlorophyll a fluorescence and oxygen evolution. As shown in Figure 3F and Supplemental Table 1, the maximal photosynthetic rate ($P_{\text{max}}$) determined by chlorophyll fluorescence decreased by about half after 3 h of HS and to ~20% of initial values after 24 h of HS. After 8 h recovery, $P_{\text{max}}$ had recovered only...
to $\sim 34\%$ of pre-HS values. By contrast, the initial slope ($\alpha$-slope) of the photosynthesis-irradiation (P-I) curve, estimating PSII quantum yield, did not change during the first 12 h of HS but dropped by $\sim 25\%$ after 24 h of HS and increased slowly during recovery (Figure 3G). In line with the $\alpha$-slope values, oxygen evolution did not change in cells exposed to HS for 3 h, but decreased by about two-thirds in cells exposed to HS for 24 h (Figure 3H; Supplemental Table 1). Hence, chlorophyll fluorescence at higher irradiances (as indicated by $P_{\text{max}}$) appears to underestimate electron flow through PSII in heat-stressed cells, whereas at limiting light (indicated by the
a-slope) it appears to be a better measure. This is in line with earlier reports, where rates of oxygen evolution correlated better with the a-slope than with P_{max} (Geel et al., 1997; Gilbert et al., 2000). Also, respiration (oxygen consumption in the dark) did not change between unstressed cells and cells exposed to HS for 3 h, but declined by about half in cells exposed to HS for 24 h (Figure 3H; Supplemental Table 1). Finally, the chlorophyll a/b ratio declined from 2.5 to 2.15 during 24 h HS and increased again during recovery, albeit only to a value of 2.25 after 24 h recovery (Figure 3I; Supplemental Table 1).

Analysis of the Dynamics of Polar Metabolites

For the profiling of polar metabolites, cells were harvested by fast filtration and polar metabolites were extracted with cold methanol/chloroform, derivatized by methoxamination and silylation, and analyzed by gas chromatography-mass spectrometry (GC-MS) (Lisec et al., 2006; Veyel et al., 2014a). Excluding standards, 106 polar analytes were identified by matching reconstructed spectra against the Golm Metabolome Database (Kopka et al., 2005; Schauer et al., 2005; Hummel et al., 2010) using the TagFinder software (Luedemann et al., 2008). The 106 analytes represented 99 distinct metabolites mainly covering

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Photosynthesis and Respiration.}
\begin{enumerate}
\item[(A)] 77K fluorescence spectra. 77K fluorescence was recorded from Chlamydomonas cells before HS, during HS, and during the recovery phase (Re). The averages of three biological and two technical replicates are shown. Spectra are normalized to the PSII emission peak at 687 nm (dotted line). The color code used is applied throughout this figure.
\item[(B)] Close-up of PSII emission peak normalized 77K fluorescence spectra. The dotted line indicates an additional emission peak occurring in heat-stressed cells at 681 nm.
\item[(C)] Relative changes in PSII emission peak normalized spectra at 681 nm. Shown is the relative change in fluorescence emission at 681 nm during HS and recovery relative to that under pre-HS conditions (0 h HS). Averages and standard deviations of three biological and two technical replicates are shown.
\item[(D)] Close-up of PSI emission peak normalized 77K fluorescence spectra. Blue shift of PSI emission peaks. Shown is the blue shift of PSI emission peaks during the HS and recovery time course based on averages of three biological and two technical replicates. Error bars represent standard deviations.
\item[(E)] Photosynthesis-irradiance (P-I) curve based on fluorescence. Electron flow through PSII was determined for cells sampled during the HS and recovery time course by PAM measurements and is represented as Y'Y'PAR (photosynthetically active radiation) plotted against the PAR applied. Error bars represent standard deviations of three biological replicates. For numerical values, see also Supplemental Table 1.
\item[(F)] a-Slopes of P-I curves. The initial slopes (a-slopes) of P-I curves from (F) were determined. Error bars represent standard deviations of three biological replicates. For numerical values, see also Supplemental Table 1.
\item[(G)] P-I curves based on oxygen evolution. Electron flow through PSII was determined for cells sampled during the HS and recovery time course by determining oxygen evolution with an optical oxygen measurement system. Error bars represent standard deviations from three biological and three technical replicates. For numerical values, see also Supplemental Table 1.
\item[(H)] Chlorophyll a/b ratios. Chlorophyll a and b concentrations were determined spectrophotometrically from three biological and two technical replicates. For numerical values, see also Supplemental Table 1. Error bars represent standard deviations.
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organic acids, amino acids, polyols, amines, sugars, phosphorylated sugars, and several unknown metabolites. After filtering, we tested 85 metabolites for significance in a one-way-ANOVA (Benjamini-Hochberg corrected P value < 0.05) resulting in 43 polar metabolites that changed significantly during the HS and recovery time course (Figure 4; Supplemental Figure 3 and Supplemental Data Set 1).

Using hierarchical clustering, polar metabolites were grouped into four clusters according to their behavior during the HS and recovery time course (Figure 4A). Five metabolites (light-blue cluster) peaked early within the first 3 h of HS, their abundance declined to levels still higher than under pre-HS conditions after 24 h HS, and in most cases decreased further during recovery. Abundances of 11 metabolites (dark blue cluster) increased steadily during HS and declined during recovery. Most of them displayed a small peak during the first hour of HS followed by a transient decline during the next 2 h before the overall increase to maximum abundances reached after 24 h HS. Abundances of 12 metabolites grouped into the yellow cluster peaked transiently during the first 3 h of HS, declined again within the next 21 h of HS (in most cases below initial levels), and often increased mildly during recovery. Finally, abundances of 15 metabolites (red cluster) declined during the first 3 h of HS, remained low during the entire duration of HS, and rapidly rose again during recovery, often to levels much higher than present under pre-HS conditions.

Principal component analysis on polar metabolite data revealed a functional and temporal separation of the metabolic state of the cells (Figure 4B). The PCA projection space factoring in the HS time points is populated almost exclusively by early-peaking metabolites (light-blue and yellow clusters) that largely appear to be generated by the degradation of larger molecules (e.g., glycerophosphorylglycerol [GPG], ethanolamine phosphate, and inositol from phospholipids; guanosine and β-alanine from nucleotides; putrescine and ornithine from arginine; see Discussion). By contrast, the projection space factoring in the recovery time points is populated by metabolites whose abundance decreased or increased only mildly during the first 3 h of HS (blue and red clusters) and represent many metabolites required for anabolic reactions (e.g., amino acids: glycine, asparagine, threonine, serine, isoleucine, and phenylalanine; central metabolites: glucose-6-phosphate, fructose-6-phosphate, glycerol-3-phosphate, citrate, malate, succinate, and 2-oxoglutarate; see Discussion). Hence, PC2, accounting for 22.75% of the variance, largely appears to separate "catabolism"-derived metabolites from those involved in "anabolism." While PC1 could not be interpreted biologically and likely represents technical noise, PC3, accounting for 15.5% of the variance, largely appears to separate metabolites required for "stress acclimation" from those involved in "housekeeping." Accordingly, the upper part of the projection space is populated by metabolites accumulating during 24 h of HS (light-blue and blue clusters) that are likely to play roles as compatible solutes (GPG, trehalose, inositol, and several amino acids; see Discussion), while the lower part is populated by metabolites exhibiting low levels after 24 h of HS (red and yellow clusters), of which many are involved in central metabolism (e.g., fumarate, succinate, malate, and citrate).

Accordingly, the trajectory of the early time points after onset of HS appears to be driven by "stress acclimation through catabolism" (i.e., generation of compatible solutes by degradation of larger molecules), while at later time points during HS it is driven by "stress acclimation through anabolism" (i.e., generation of compatible solutes by de novo synthesis; see Discussion). The trajectory during recovery appears to be driven by "restoration of housekeeping metabolism" (i.e., removal of compatible solutes and overshooting of metabolites from the central metabolism). Notably, PCA also revealed that the cellular metabolic state after 8 h recovery was still different from that under pre-HS conditions.

**Analysis of Proteome Dynamics**

Proteome dynamics during heat stress and recovery was monitored by applying a shotgun proteomics approach based on full 15N metabolic labeling. For this, cells were 15N-labeled to >98% by growth on 15NH4Cl. Unstressed and heat-stressed labeled cells were pooled to generate a 15N standard for each protein expressed during the HS and recovery time course, which was then mixed with 14N cells harvested at different time points during the experiment (Figure 1). Membrane and soluble proteins were extracted, tryptically digested, and subjected to nano-liquid chromatography-tandem mass spectrometry. Proteins were identified and quantified with the IOMiQS framework (integration of mass spectrometry identification and quantification software) (Mühlhaus et al., 2011). With this approach, a total of 1985 proteins were quantified in samples from at least five out of six time points taken during HS and recovery. As for 181 of them, no unique peptides were identified, and they were combined into 52 protein groups. Hence, we quantified 1856 proteins/protein groups, which for simplicity will be designated as "proteins" in the following (Supplemental Data Set 2). Of these, 688 could be determined as significantly changing within the time course using one-way ANOVA with a P value threshold of 0.05 after correction for multiple testing (Benjamini and Hochberg, 1995).

Using hierarchical clustering, the 688 changing proteins were grouped into five clusters according to their behavior in the time course (Figure 5A; Supplemental Figure 4). To elucidate whether proteins sharing a similar behavior were enriched in certain functional categories, a functional enrichment based on the MapMan ontology (Thimm et al., 2004; Lohse et al., 2014) was calculated (Supplemental Data Set 3). According to this analysis, proteins whose abundance increased strongly during HS and decreased during recovery (light blue) are enriched in functional categories photosystems, exotic lipid metabolism, stress, redox, protein degradation, protein folding, vesicle transport, and glycolysis. Proteins whose abundances increased slightly during HS and strongly in the initial recovery phase (dark blue) are enriched in categories N metabolism, amino acid and nucleotide synthesis, translation initiation, and protein degradation. Proteins whose abundance declined strongly during HS and increased slowly during recovery (red) were enriched in categories mitochondrial and chloroplast ATP synthases, Calvin cycle, N metabolism, amino acid degradation, tetrapyrrole synthesis, DNA synthesis, translation, and gluconeogenesis/glyoxylate cycle. Proteins whose abundances decreased during HS and recovered afterwards (yellow) are enriched mainly in categories protein synthesis and chloroplast protein targeting. Members of the gray cluster hardly changed during HS, but decreased during recovery. This
Figure 4. Polar Metabolite Data.

(A) Cluster behavior and cluster tree of polar metabolites. The behavior of the four clusters was determined by hierarchical clustering combined with gap statistics on log$_2$-transformed polar metabolite data. The shift from HS to recovery is hallmarked by a dotted line. The colors of branches in the cluster tree indicate the affiliation of metabolites to one of the four clusters.

(B) PCA on polar metabolite data. Log$_2$-transformed data from all 43 metabolites found to be changing during the HS and recovery time course ($P \leq 0.05$) were included. Time points are represented by dotted circles and individual metabolites by rhombi. The color of a rhombus indicates its affiliation to one of the four clusters displayed in (A).
PCA applied to the proteome data revealed that the main variance (PC 1, 67.38%) clearly separates the cellular proteome compositions (referred to as proteome states) before and after HS treatment, especially time points 0 h HS from 24 h HS (Figure 5A). Separation along PC 1 mainly can be attributed to proteins upregulated (light and dark blue) and downregulated (red and yellow) during HS that respectively contribute to opposing vectors. Specifically, functional categories like stress, protein folding, photosystems, redox, glycolysis, or protein degradation oppose categories like gluconeogenesis/glyoxylate cycle, Calvin cycle, DNA synthesis, tetrapyrrole synthesis, N metabolism, protein synthesis, and ATP synthases (Supplemental Data Set 3). Hence, this separation appears to indicate a functional partitioning between “stress acclimation” and “housekeeping” proteome states. Although PC 3 accounts for only 8% of the total variance, we observe a separation between proteins in the light-blue, gray, and red clusters on the left and dark-blue and yellow clusters on the right side. As we find proteins enriched in categories N metabolism, amino acid biosynthesis, nucleotide synthesis, and protein synthesis in the dark-blue and yellow clusters, observations on the protein level appear to correlate with the separation between “catabolic” and “anabolic” processes, as also apparent from the metabolite analysis.

PCA also revealed the relative cellular proteome compositions prior to HS, 24 h after HS, and 8 h after recovery to be the most uncorrelated time points in the data set (Figure 5A). Proteome states in cells after 1, 2, and 4 h of recovery were found to be on the trajectory between 24 h HS and 8 h recovery; therefore, the proteome state at 8 h recovery contains most information present in the other recovery time points. Accordingly, we generated three different contrasts (24 h versus 0 h HS, 8 h recovery versus 24 h HS, and 8 h recovery versus 0 h HS) to compare proteome states representative for nonstress, prolonged heat stress, and recovery in more detail (Figure 5B). When comparing proteome states of 24 h HS with unstressed cells, we found seven functional categories to be significantly enriched with differentially expressed proteins. Four of them contain proteins that were mainly upregulated (branched chain amino acid metabolism with four proteins upregulated; abiotic heat stress with five proteins upregulated; redox with 21 proteins upregulated and two downregulated; and protein folding with 34 proteins upregulated and two downregulated) (Supplemental Data Set 2, Note 1). Two functional categories contain proteins that were almost entirely downregulated (histones with 15 proteins downregulated; ribosomal proteins with 51 proteins downregulated and one upregulated). The functional category photosynthesis is a split category containing 33 upregulated and 23 downregulated proteins with no clear separation between functions.

A comparison of the proteome states between 8 h recovery and 24 h HS revealed six functional categories to be enriched with differentially expressed proteins (Figure 5B; Supplemental Data Set 2, Note 1). The very large part of these in categories photosynthesis, redox, histones, ribosomal proteins, and protein folding changed in the opposite direction of that with which they had changed during 24 h of HS or underwent no significant change. By
contrast, all proteins involved in branched chain amino acid synthesis continued to increase or remained upregulated.

When comparing proteome states of cells that had recovered for 8 h from 24 h of HS with unstressed cells (0 h HS), eight functional categories were still found to be enriched with differentially expressed proteins, although cells were again competent for dividing in this state (Figure 5B; Supplemental Data Set 2, Note 1). A very large portion of proteins in categories photosynthesis, redox, abiotic stress/protein folding, histones, and branched chain amino acid/aspartate synthesis were downregulated in the direction into which they had changed during the 24 h of HS and only few had again reached pre-HS levels. While only very few proteins in these categories had changed into a direction opposite to that into which they had changed during HS, this was the case for most ribosomal proteins. Hence, when compared with unstressed cells, almost all proteins in bins redox, abiotic stress/protein folding, branched chain amino acid/aspartate synthesis, and ribosomal proteins were upregulated in cells that had recovered for 8 h from 24 h of HS. Histones were downregulated and photosynthesis-related proteins showed mixed behavior.

Analysis of Dynamics of Lipophilic Metabolites

Lipophilic metabolites were analyzed using ultraperformance liquid chromatography and high-resolution mass spectrometry (Hummel et al., 2011). We profiled 185 distinct lipophilic compounds by extracting exact masses from the ultraperformance liquid chromatography and high-resolution mass spectrometry chromatograms for respective metabolites. Of the 185 compounds, 108 represented metabolites from six of the seven major membrane lipid classes present in Chlamydomonas: diacylglycerol trimethyl homoserine (DGTS; a primary betaine membrane lipid in Chlamydomonas considered to replace phosphoryl cholin; Giroud et al., 1988) and phosphoryl ethanolamine (PE) classified as cytoplasmic lipids; monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG) classified as plastidic lipids; and phosphoryl glycerol (PG) being present in both compartments (Janero and Barrnett, 1981). In addition, nine diacylglycerol (DAG) species and 68 TAG species were monitored. Neither phosphorylated inositol nor phosphoryl cholin was detected with the applied method. Out of the 185 lipophilic metabolites, abundances of 164 changed significantly during HS and recovery, among these, ~86.1% (93 of 108) of the detected membrane lipid species and ~98.5% (67 of 68) of the TAGs (P value ≤ 0.05) (Supplemental Figure 5 and Supplemental Data Set 4).

Based on their behavior in the HS and recovery time course, TAGs were grouped into two clusters by hierarchical clustering (Figure 6A; Supplemental Figure 6). Abundances of TAGs belonging to the red cluster increased strongly during the first 2 h of HS, declined slightly during the next 22 h of HS, and declined strongly during recovery. Fatty acids (FAs) of these TAGs contained a total of 52 to 56 carbons and 4 to 10 double bonds (Figures 6B and 6C). Abundances of TAGs belonging to the blue cluster increased gradually during HS and recovery. They were enriched in species containing 0 to 6 double bonds and 48 to 50/54 to 60 carbon atoms. In PCA, these two clusters are separated along PC 1 (49.57%) (Figure 6D), most likely reflecting a different source of FAs incorporated into the TAGs of the two clusters (see Discussion).

The data set on membrane lipids is far less complete and much more complex than that on TAGs. Based on the behavior of lipids during the HS and recovery time course, hierarchical clustering allowed their grouping into five clusters (Figures 7A and 7B). Abundances of lipids in the red cluster increased rapidly after the onset of HS, remained high during HS, and decreased during recovery. Lipids in the light blue cluster behaved opposite to lipids in the red cluster as their abundance decreased rapidly after onset of HS, remained low during HS, and increased during recovery. Abundances of lipids in the yellow cluster decreased during the first 2 h of HS, but increased during the remaining 22 h of HS and during recovery. The opposite behavior was observed for lipids in the dark-blue cluster, which increased during the first 2 h of HS, but declined during the remaining 22 h of HS and during recovery. Abundances of lipids grouped into the cyan cluster continuously decreased during HS and increased in the recovery phase.

FAs in lipids of the upregulated red and yellow clusters were generally shorter and more saturated than FAs in lipids of the downregulated (light blue, dark blue, and cyan) clusters (Figures 7C and 7D). Regarding the distribution of individual lipid species among the clusters, we observe a strong enrichment of cytosolic membrane lipid DGTS in the red cluster (10 of 11 lipid species). While only a single plasticid lipid species (DGDG_34.0) is present in the red cluster, six other plasticid lipids (one MGDG, four DGDGs, and one SQDG) with 0 to 2 double bonds are present in the yellow cluster, suggesting that the increase in these plasticid lipids with more saturated FAs occurs strongly delayed if at all when compared with cytosolic DGTS. Differences in cluster behavior and cluster composition also are well separated by PCA, where upregulated (red and yellow) and downregulated (light blue, dark blue, and cyan) lipids appear separated along PC 1 (61.2%), whereas cytosolic (enriched in red and dark blue clusters) and plasticid lipids (enriched in yellow and light blue clusters) appear separated along PC 2 (22%) (Figure 7E).

Overall, membrane lipids show a trend opposite to that of TAGs. In particular, abundances of cytosolic DGTS species with shorter chain lengths and more saturated FAs increase during the first 2 h of HS, while abundances of lipids of all classes with longer and more unsaturated FAs decrease. By contrast, TAGs with more unsaturated FAs drastically increase during the same time frame and decrease during recovery. Strikingly, although cells commenced dividing again after 8 h recovery, their lipid composition was still much different from that present before HS (Figure 7E; Supplemental Figure 5).

DISCUSSION

Using a top-down systems biology approach, we addressed the question of how Chlamydomonas acclimates to long-term HS and recovers from it. The HS and recovery time course shows changes in molecular compositions over time allowing Chlamydomonas to acclimate to the elevated temperature and back to ambient temperature. This acclimation process is characterized by specific response elements grouped by a common functional and temporal behavior. In the following, we will
Discuss these response elements and their dynamic succession during the HS and recovery time course.

**Cells Undergo an Immediate Cell Cycle Arrest upon Onset of HS**

A characteristic early response to HS is an immediate arrest of cell division (Figures 2A and 8), which we have observed previously in *Chlamydomonas* and which is conserved in eukaryotic cells (Richter et al., 2010; Mühlhaus et al., 2011). As the distribution of 1n, 2n, and 4n cells remained almost constant during HS (Figure 2B), cells apparently were cell cycle arrested in both G1 (unreplicated DNA = 1n) and G2 phases (after DNA replication $= 2n, 4n$), perhaps as a consequence of reduced activities of cyclin-dependent kinases (Kühl and Rensing, 2000).
Figure 7. Membrane Lipid and DAG Data.
biosynthesis of histones is restricted to the S phase (Walther and Hall, 1995; Marzluff and Duronio, 2002), no de novo biosynthesis of histones is expected to occur in heat-stressed Chlamydomonas cells. Accordingly, we find levels of histone proteins to decline on average by ~30% after 24 h of HS, which is close to the expected dilution by ~36% given the 57% increase in cellular protein content in this time period (Figure 2D).

**Metabolic Remodeling Early after Onset of HS Is Characterized by the Depletion of Central Metabolites**

An early response to HS is the remodeling of the cell’s metabolic state. Part of this is the rapid depletion of metabolites from primary metabolism, including succinate, malate, 2-oxoglutarate, and citrate of the TCA and glyoxylate cycles, or fructose-6-phosphate, glucose-6-phosphate, and glyceraldehyde-3-phosphate as metabolites of glycolysis and Calvin cycle (Figures 4 and 8; Supplemental Figure 3 and Supplemental Data Set 1). Although several TCA/glyoxylate cycle and glycolysis enzymes were significantly downregulated during the first 3 h of HS (Mühlhaus et al., 2011; “short-term HS (3 h HS versus 0 h HS)” in Supplemental Data Set 2, Note 1), the degree of downregulation was so modest that depletion of corresponding metabolites most likely is due to reduced enzyme activity. In Escherichia coli, HS also led to a rapid depletion of metabolites from glycolysis, the oxidative pentose phosphate pathway, and the TCA cycle and was interpreted as part of an energy conservation strategy (Jozefczuk et al., 2010). By contrast, metabolites of glycolysis and Calvin cycle were found to increase in heat-stressed Arabidopsis (Kaplan et al., 2004; Rizhsky et al., 2004). Further work is required to elucidate whether this discrepancy is related to trophic state (mixo/heterotrophy versus autotrophy), organization (single versus multicellularity), or generally different HS acclimation strategies between these organisms.

**Metabolic Remodeling Early after Onset of HS Is Also Characterized by the Accumulation of Metabolites Potentially Involved in Stress Protection**

Although from their overall behavior during the HS and recovery time course belonging to three different clusters (light blue, blue, and yellow in Figure 4), 12 metabolites had in common to strongly accumulate within the first 2 h of HS and to contribute significantly to the factoring of the early time points in the projection space of the PCA (representing “catabolism”) (Figure 4B; Supplemental Figure 3 and Supplemental Data Set 1). These are GPG, guanosine, ethanamine phosphate, β-alanine, myo-inositol, glutamate, pyroglutamate, ornithine, a hexo-aldose, putrescine, fumarate, and trehalose.

The rapid increase of β-alanine appears to be a conserved metabolic signature for HS in a wide range of organisms, as it has been observed also in E. coli (Jozefczuk et al., 2010), Arabidopsis (Kaplan et al., 2004; Rizhsky et al., 2004), and Drosophila melanogaster (Malmendal et al., 2006). β-Alanine may be synthesized from polyamines (Terano and Suzuki, 1978) or propionate (Hatch and Stumpf, 1962) or may derive from the degradation of uracil (Duhaze et al., 2003). The notion that uracil from degraded RNA is the source of β-alanine appears supported by the observation that guanosine, a breakdown product of guanine nucleotides, accumulated with similar kinetics as β-alanine. Accumulating β-alanine was suggested to serve as a compatible solute (Kaplan et al., 2004). Compatible solutes stabilize membranes and prevent protein unfolding and aggregation (Singer and Lindquist, 1998b; Martin et al., 1999; Yancey, 2005). However, β-alanine also is a precursor for the biosynthesis of pantothenate (vitamin B5), which is required for the biosynthesis of the 4′-phosphopantetheine moiety of CoA and acyl carrier protein (Ottenhof et al., 2004). An increased requirement of the latter for FA metabolism immediately after onset of HS is an attractive idea given the dramatic lipid rearrangements taking place early after onset of HS (see below).

The metabolite with the most striking increase within the first 2 h of HS was GPG (Supplemental Figure 3A and Supplemental Data Set 1). In fact, GPG was found to accumulate in response to salt stress and elevated temperatures in the thermophilic archaeabacterium Archaeoglobus fulgidus (Martins et al., 1997) and to exhibit a thermostabilizing effect on several proteins in vitro (Lamosa et al., 2000, 2003; Santos and da Costa, 2002). As our GC-MS measurements provide only relative data on GPG levels, we cannot assess whether the absolute concentrations of accumulating GPG are sufficiently high for a compatible solute.
function in *Chlamydomonas*. GPG may be produced by the actions of phospholipase A or lipid acyl hydrolase, which degrade PG into GPG and free FAs (Matos and Pham-Thi, 2009; Cheng et al., 2011). Accordingly, we found levels of all PG species to decrease already after the first hour of HS (Figure 7B; Supplemental Figure 5).

With ethanolamine phosphate and myo-inositol, two additional possible degradation products from phosphorous glycolipids increased early after onset of HS (Supplemental Figure 3B and Supplemental Data Set 1). Ethanolamine phosphate is the head group of PE and myo-inositol that of phosphatidyl inositol (deprived of the phosphate moiety). These metabolites might be produced by phospholipases C and D, respectively (Matos and Pham-Thi, 2009), which is in agreement with a decrease already after the first hour of HS in PE species whose FAs contain three or more double bonds (Figure 7B; Supplemental Figure 5). In contrast to GPG, but similar to β-alanine, levels of ethanolamine phosphate and myo-inositol reached their maximum already within the first 15 min of HS and declined afterwards, but remained elevated during HS. Whether they serve in signaling (Mishkind et al., 2009), as compatible solutes (Yancey, 2005), or are an inert by-product of lipid remodeling remains to be elucidated.

Sugars like sucrose, rafinose, and maltose increased early after onset of HS in *Arabidopsis* and were suggested to serve as compatible solutes (Kaplan et al., 2004; Guy et al., 2008). Likewise, early HS-induced hexo-aldose and trehalose might act as compatible solute in *Chlamydomonas*. Possibly, a similar role can be attributed to pyroglutamate, as it has been shown to serve as osmoregulant in haloalkaliphilic methanotrophs (Trotsenko and Khelenina, 2002). However, as pyroglutamate may be generated by glutamate cyclization and shares a very similar kinetic during HS and recovery with glutamate (Supplemental Figure 3B), it might also be an experimental artifact (Steinhauser and Kopka, 2007).

Ornithine and putrescine are likely generated from the degradation of arginine in a pathway leading to the biosynthesis of spermidine from putrescine (Kusano et al., 2008). This assumption is supported by the 2.1-fold increase in abundance of spermidine synthase SPD1 after 24 h HS (Supplemental Data Set 2). When exogenously applied, spermidine was shown to protect *Arabidopsis* seedlings from HS (Sagor et al., 2013).

Overall, the early response to HS at the metabolite level is characterized by the rapid accumulation of compounds that most likely are generated rapidly because they are derived from the catabolism of larger molecules (Figure 8). These products from catabolism may represent precursors for the biosynthesis of compounds playing roles in stress protection (β-alanine for pantothenate biosynthesis; ornithine and putrescine for spermidine biosynthesis) or function directly in stress protection as compatible solutes [GPG, inositol, trehalose, and (pyro)glutamate].

**Membrane Lipid Remodeling Is an Early Response to HS and Appears to Occur with Lipid Bodies as Buffer for Unsaturated Fatty Acids**

Within the first 2 h of HS, strong rearrangements in the composition of lipophilic metabolites took place. These rearrangements were dominated by the accumulation of DAGs and TAGs with polyunsaturated FAs, the accumulation in particular of cytosolic DGTS with saturated FAs, and the decrease in abundance of lipids with polyunsaturated FAs (Figures 6 to 8; Supplemental Figure 5). While abundances of cytoplasmic and plastidic lipids with polyunsaturated fatty acids decreased to a similar extent, we observed a much slower increase of more saturated plastidic lipids as compared with cytosolic DGTS (compare red with yellow cluster in Figures 7A, 7B, and 7E). A possible explanation for this discrepancy might be that plastidic membrane lipids with polyunsaturated FAs are not replaced by saturated species of the same lipid class, but by other molecules not captured in our analysis. These may comprise specific saturated stress lipids like MGlicDG found in cyanobacteria to accumulate under heat stress conditions and, together with small heat shock proteins, to preserve the functional integrity of thylakoid membranes (Tsvetkova et al., 2002; Balogi et al., 2005). To accomplish lipid remodeling, lipid acyl hydrolases and phospholipases apparently target mainly membrane lipids containing polyunsaturated FAs, leading to the generation of DAGs and free FAs, which are converted into TAGs. The dark staining of lipid bodies in electron micrographs taken 3 h after onset of HS, apparently caused by the strong interaction of osmium tetroxide with double bonds generating higher electron density (Hayat, 1970), corroborates that TAGs in these lipid bodies contain polyunsaturated FAs (Figure 2F).

Lipids with saturated FAs are generated by the de novo biosynthesis of saturated palmitic (16:0), stearic (18:0), and monounsaturated oleic (18:1) FAs in the chloroplast (Ohlrogge and Browse, 1995). Additional double bonds are introduced later via different desaturases (Riekoff et al., 2005; Hu et al., 2008). The introduction of further carbon atoms (two per cycle) to elongate FAs is achieved even later by membrane-bound elongation enzymes situated in the ER membrane (Nugteren, 1965; Ghanevati and Jaworski, 2001). Accordingly, we found the saturated FAs in lipids increasing during the first 2 h of HS on average to be shorter than those of the declining lipids with polyunsaturated FAs (Figures 7A to 7C; Supplemental Figure 5), in line with the proposed de novo biosynthesis of lipids with saturated FAs. De novo biosynthesis of lipids with saturated FAs requires glycercol, acetyl-CoA, NADPH, and ATP (Ohlrogge and Browse, 1995). As during the first 2 h of HS the increase in TAGs and lipids with saturated FAs correlates with a decrease in starch (Figure 2E), glycerol and acetyl-CoA might to some part derive from starch breakdown, but acetate from the medium is also a likely source. As discussed below, the main portion of ATP and NADPH might be contributed by the light reactions of photosynthesis, whose capacity was found not to be impaired 3 h after onset of HS (Figure 3H).

What may be the reason for the HS-induced rapid increase in the fraction of membrane lipids with saturated FAs? The ratio of saturated to unsaturated FAs in membrane lipids was shown to correlate with growth temperature in poikilothermic organisms (Marr and Ingraham, 1962, and references therein). This ensures a constant membrane viscosity to ensure functionality of membrane proteins and was termed “homeoviscous adaptation” (Sinesky, 1974). Homeoviscous adaptation to HS is fast and in bacteria can be accomplished within 30 min after
onset of HS (Mejía et al., 1995; Shigapova et al., 2005; Li et al., 2013). Our results suggest that homeoviscous adaptation also occurs within a few hours in heat-stressed Chlamydomonas cells.

While the fraction of lipids with unsaturated FAs may increase by de novo biosynthesis and the activity of desaturases (Russell, 1984), de novo biosynthesis is the only option for increasing the fraction of lipids with saturated FAs. This implies the exchange of unsaturated FAs by de novo produced saturated FAs and, given the cell cycle arrest of heat-stressed Chlamydomonas cells, the necessity to dispose of unsaturated FAs by means other than dilution by growth. Apparently, by employing lipid bodies as provisional sinks for unsaturated FAs in form of TAGs, Chlamydomonas has found an elegant solution to this problem and it is likely that this applies also to other stresses like N or S deprivation (Sheehan, 1998; Miller et al., 2010; Cakmak et al., 2012). However, as discussed below, TAGs in light of the cell cycle-arrested cells should also be considered as sinks for NADPH, ATP, and fixed carbon from photosynthesis; in cases of nutrient stress, this may be their dominating role. Interestingly, exposure of Arabidopsis to a 30-min HS at 40°C led to a significant increase in the number of plastoglobules (Zhang et al., 2010). Plastoglobules are attached to thylakoids through a half-lipid bilayer that surrounds the globule and is continuous with the thylakoid membrane (Austin et al., 2006). They contain lipids, FAs, TAGs, carotenoids, tocopherols, quinones, and chlorophylls and have been shown to accumulate also under other conditions likely to require lipid remodeling, like deetiolation, senescence, or salt stress (Sprey and Lichtenthaler, 1966; Kaup et al., 2002; Sam et al., 2003). Hence, it is tempting to speculate that analogous to lipid bodies in Chlamydomonas, plastoglobules may serve as provisional deposits for FAs from membrane lipid remodeling and as sinks for NADPH, ATP, and carbon in stressed land plant chloroplasts.

**Levels of Molecular Chaperones Are Modulated between 3 and 24 h HS Depending on Protein Class and Subcellular Localization**

Prolonged HS faces cells with the problem of enhanced rates of protein unfolding for which they have to compensate to reestablish protein homeostasis at the elevated temperature (Sharma et al., 2011). This may be achieved by a combination of elevated

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**Figure 8. Cellular Responses during the HS and Recovery Time Course.**

Relative activities of cellular processes or abundances of cellular compounds are given as bars, with bar thickness being roughly proportional to activity/abundance. Dotted lines indicate the absence of an activity/compound.
levels of molecular chaperones, reduced expression of (thermolabile) proteins, and the accumulation of compatible solutes (Singer and Lindquist, 1998a, 1998b). Accordingly, except for the Hsp110 family member HSP70G, all proteins in bins protein folding (29.6) and abiotic stress (bin 20.2.1), previously found to increase early after onset of HS, were still upregulated after 24 h of HS (Figures 5B and 8; Supplemental Data Set 2, Note 1; Mühlhaus et al., 2011). Except for two universal stress proteins, all proteins in these bins are (co)chaperones and immunophilins. However, there was variation between levels reached after 3 and 24 h of HS that depended on the (co)chaperone class or subcellular location. For example, levels of small HSPs and ClpB-type HSP100s declined between 2.3- and 7.1-fold after 24 h HS compared with 3 h HS (Supplemental Data Set 2, Note 1, and Supplemental Figure 7). sHSPs intercalate into forming protein aggregates to improve their accessibility for ClpB/HSP70 and ClpBs mediate the disassembly of protein aggregates (Goloubinoff et al., 1999; Mogk et al., 2003; Weibelzahn et al., 2004). Hence, the formation of aggregates appears to be reduced in HS-acclimated cells, presumably mediated by the accumulation of compatible solutes, potentially including GPG, trehalose, a hexasialoside, inositol, and several amino acids. Moreover, as levels of ClpB/ sHSPs declined by significantly more than 29% (expected from dilution resulting from the 40% increase in total cellular protein content between 3 and 24 h HS), they appeared to be actively degraded, thus indicating a tight control of their levels.

Examples for compartment-specific long-term regulation of chaperone levels are the ER-resident BIP1 and HSP90B, which increased only after 24 h HS or continued to increase, respectively, and mitochondrial HSP70C and CPN60C/CPN10 that continued to increase between 3 and 24 h HS (Supplemental Data Set 2, Note 1, and Supplemental Figure 7). Apparently, processes in ER and mitochondria taking place late after onset of HS require more tuning of chaperoning power to meet the chaperoning requirements of specific substrates in the new steady state.

**Reduced Bulk Protein Biosynthesis during HS Comes Along with Reduced Abundances of Cytosolic, but Not Plastidic, Ribosomes**

The generation time of exponentially growing *Chlamydomonas* cells of 5 to 8 h implies that bulk protein per culture volume under optimal conditions increases by at least 8-fold in 24 h (Harris, 2008). However, over the 24-h HS period, we observed only a 57% increase in bulk protein per cell (Figure 2D); as cell numbers/mL remain constant during HS (Figure 2A), the 57% increase in bulk protein is valid also based on culture volume. Hence, HS leads to a reduction of general protein synthesis in *Chlamydomonas* that persists during the entire HS period, although after onset of HS HSPs are biosynthesized at very high rates (Schulz-Raffelt et al., 2007; Schmollinger et al., 2013). This reduced need for translation capacity manifests itself also by a ~17% decrease in cytosolic ribosome content, which was already observed after 3 h HS and persisted during the 24-h HS period (Figure 5B; Supplemental Data Set 2, Note 1; Mühlhaus et al., 2011). A decrease in ribosome content under HS was also observed in yeast, where it is realized by the heat shock factor-dependent downregulation of genes encoding ribosomal proteins (Lopez et al., 1999). Interestingly, in contrast to the 45 significantly declining cytosolic ribosomal proteins, levels of only two organellar ones declined mildly (by 8 and 15%) after 24 h of HS. Moreover, a single ribosomal protein, termed plastid-specific protein 1 (PSRP-1), increased 1.3-fold during the 24-h HS period (Figure 5B; Supplemental Data Set 2, Note 1). PSRP-1 contains a ribosome-associated inhibitor A (RaiA) domain. Bacterial RaiA (or pY) inhibits translation initiation upon cold stress by stabilizing 70S ribosomes and preventing aminoacyl-tRNA and mRNA binding (Vila-Sanjurjo et al., 2004). This indicates the possibility that plastidic translation during HS might be throttled by PSRP-1.

**Only 29 Proteins Are Found to Be Actively Degraded during Long-Term HS**

Proteins that cannot be folded and associate with chaperones for too long are handed over to proteases for degradation (Arndt et al., 2007). The 57% increase in total cellular protein during the 24-h HS period corresponds to a dilution by ~36%. Hence, degradation rates of proteins declining significantly more than 36% must exceed their biosynthesis rates. Surprisingly, this is the case only for 29 of the 688 proteins changing significantly during the HS and recovery time course (Supplemental Data Set 2, Note 2). These are likely to include (1) severely heat labile proteins, (2) proteins exerting functions that jeopardize survival during long-term HS, and (3) proteins only transiently required during HS. A potential example for heat labile proteins is METE, which was downregulated by 59% after 3 h and by 71% after 24 h of HS (Supplemental Data Set 2, Note 2; Mühlhaus et al., 2011). Accordingly, *E. coli* MetE was found to denature and aggregate upon HS, to be solubilized by the ClpB/DnaK chaperones, and, upon lasting HS, to be degraded by the Lon protease (Mogk et al., 1999). An example for undesired proteins might be CTH1B and chlB, enzymes involved in chlorophyll biogenesis, which decreased by 49 and 81%, respectively, after 24 h HS (Supplemental Data Set 2, Note 2). Chlorophyll biosynthesis might be throttled to avoid potentially improperly assembled chlorophyll that would produce reactive oxygen species (ROS) (Mittler, 2002; Stenbaek and Jensen, 2010). Examples for transiently required proteins are THI4 and THI8, which are involved in thiamine biosynthesis and decrease by 62 and 85% respectively. As THI4 was found to increase 1.5-fold after 3 h HS (Mühlhaus et al., 2011), a demand for thiamine appears to prevail only early after the onset of HS. Similarly, the homolog of THI4 was upregulated after 6 h HS and downregulated after 54 h HS in poplar (Ferreira et al., 2006).

**Proteins Upregulated during HS Are Enriched in Branched Chain Amino Acids**

Four proteins in bin branched-chain amino acid (BCAA) synthesis were significantly upregulated after 24 h HS, although
members of this functional category were downregulated or did not change significantly after 3 h of HS (Figure 5B; Supplemental Data Set 2, Note 1; MühHaus et al., 2011). Hence, upregulation of enzymes involved in BCAA biosynthesis qualifies as a late response to HS and is consistent with the increase in levels of leucine and isoleucine during HS (Figure 4; Supplemental Figures 3A and 3B and Supplemental Data Set 1). This is in line with a previous study on heat-stressed poplar, where a ketol-acid reductoisomerase (AAI1) involved in BCAA biosynthesis was upregulated after 6 h HS (Ferreira et al., 2006). As this enzyme was also upregulated in heat-stressed Agrobacterium tumefaciens, it appears to be a conserved response (Rosen et al., 2002). Ferreira et al. suggested that proteins biosynthesized de novo during HS might contain more BCAA to better protect them from ROS attack, as BCAA are less prone to oxidation. Alternatively, proteins newly biosynthesized during HS may be equipped with a more elaborate hydrophobic core, rendering them more thermostable and their folding less chaperone dependent (Pace et al., 1996). Both ideas are supported by the finding that BCAAs are significantly enriched in proteins upregulated during HS (Supplemental Figure 8) and that these comprise upregulated ROS scavengers, as well as all HSP100s, HSP90s, HSP70s, HSP60s, and HSP10s (but not the sHSPs) (Supplemental Data Set 2, Note 3).

Antenna Uncoupling Reduces Photosynthetic Electron Flow Only under Long-Term HS

As judged from unchanged oxygen evolution rates and α-slopes of P-I curves, the capacity of the photosynthetic light reactions was not impaired in cells exposed to HS for 3 h (Figures 3G and 3H; Supplemental Table 1). Accordingly, 3 h of HS also had no effect on levels of selected subunits of PSI, PSII, LHCII, cytochrome b_{6}/f complex, or ATP synthase, and PSII maximum quantum efficiency was only slightly reduced (Nordhus et al., 2012). Also, on the basis of earlier work, no effect would be predicted on oxygen evolution in mixotrophically grown Chlamydomonas cells exposed to 42°C for 3 h (Schuster et al., 1988; Tanaka et al., 2000). PSII activity was also not affected when Arabidopsis plants were exposed for 30 min to moderate HS of 40°C (Zhang and Sharkey, 2009; Sharkey and Zhang, 2010).

By contrast, long-term HS had substantial functional and structural effects on the light reactions. These may be interpreted as a consequence of the functional uncoupling of LHCII and LHCII from the photosystems, as evidenced by the blue shifts of short- and long-wavelength 77K fluorescence signals and presumably also by their increased ratio (Figures 3A to 3E) (Naumann et al., 2005). Reduced excitation energy transfer from antenna to PS cores is likely to result in reduced linear electron flow (LEF), thus accounting for the two-thirds reduced oxygen evolution capacity and the smaller α-slope of P-I curves (Figures 3F to 3H; Supplemental Table 1). Already in an early study on the effect of increased growth temperatures on photosynthetic light reactions, the desert shrub Larrea divaricata grown at 45°C showed functional uncoupling of LHCII, an increase in the ratio of short to long wavelength 77K fluorescence signals, and a lower chlorophyll a/b ratio than 20°C-grown plants (Armond et al., 1978). As revealed by freeze-fracture of heated oleander chloroplasts, functional uncoupling of LHCII was even followed by physical uncoupling, leading to grana unstacking (Armond et al., 1980). The latter and the formation of modified thylakoid attachment sites were suggested to be caused by the migration of PSII, physically detached from LHCII, to stroma lamellae (Gounaris et al., 1984). In addition to thylakoid membrane destacking, moderate HS in Arabidopsis and turfgrass was also shown to lead to chloroplast swelling (Xu et al., 2006; Zhang et al., 2010). In transmission electron microscopy images, we observed neither after long-term HS in Chlamydomonas (Figure 2F; Supplemental Figure 2). In this respect, it is interesting to note that we found the two CURT-like proteins Cre27.g775100 and Cre10.g433950 to be upregulated 1.3- and 2.4-fold, respectively, by long-term HS (Supplemental Data Set 2). In Arabidopsis, CURT proteins induce thylakoid membrane curvature and are important for thylakoid architecture (Armbruster et al., 2013); therefore, their homologs might help preserve the latter in heat-stressed Chlamydomonas chloroplasts.

Prolamellar Body-Like Structures Appear Only under Long-Term HS and Might Consist of Unassembled Photosystems

Strikingly, between 12 and 24 h of HS, aberrant PLB-like structures occurred at nodal points between multiple thylakoid membrane layers (Figures 2F and 8; Supplemental Figure 2). PLB-like structures were identified in Chlamydomonas cells depleted of VPP1 and suggested to contain both aggregated PS subunits and assembly factors that appear at putative chloroplast thylakoid centers when PS biogenesis is disturbed (Nordhus et al., 2012; Rütgers and Schrøda, 2013). Hence, we speculate that most PSI and PSII accumulating during long-term HS is nonfunctional and buried in the PLB-like structures, as also indicated by the reduced oxygen evolution rates and the concomitant ~15% decrease in abundance of ATP synthase subunits (Supplemental Data Set 2, Note 1). PS biogenesis at thylakoid centers may be disturbed by the strong depletion after 24 h HS of many PG and SQDG species, which are structural lipids in PSI and PSII (Figure 7; Supplemental Figure 5) (Jordan et al., 2001; Sakurai et al., 2006; Guskov et al., 2009). Also a reduced efficiency of incorporation of chlorophyll into the PSs may disturb their proper assembly and might explain the decline in chlorophyll a/b ratios during HS (Figure 3I). Alternatively, as the VPP1 oligomers are dynamically assembled and disassembled by HSP70B and HSP90C, a sequestration of both chaperones to proteins constantly unfolding during long-term HS might impair VPP1 function and impair PS biogenesis (Liu et al., 2007; Heide et al., 2009; Feng et al., 2014). The strong 2.3-fold upregulation of VPP1 by long-term HS may indicate an attempt of cells to compensate for impaired VPP1 function (Supplemental Data Set 2). Whether the accumulation of PS subunits during long-term HS is caused by an enhanced synthesis or rather by the inability of clearance proteases to remove inactive/improperly assembled PSs is an open question.

Calvin Cycle Activity Might Be Reduced Early after Onset of HS to Provide Reducing Power and ATP for the Biosynthesis of Saturated FAs, but Appears to Recover under Long-Term HS

After 3 h of HS, we previously observed a 6 to 15% decrease in the abundance of Calvin cycle enzymes and of several proteins involved in carbon concentration. A remarkably stronger decrease
by 30% was found for Rubisco activase (RCA1), albeit RCA1 transcripts were increasing, indicating that, similar to its ortholog in land plants, also *Chlamydomonas RCA1* is heat labile (Feller et al., 1998; Mühlhaus et al., 2011). These data, combined with the depletion of intermediates or products of the Calvin Cycle (glycerate-3-phosphate, glucose-6-phosphate, fructose-6-phosphate, and starch), suggest that like in land plants carbon fixation by the Calvin cycle in *Chlamydomonas* is reduced during the first 3 h of HS (Weis, 1981; Kobza and Edwards, 1987).

It was suggested previously that the deactivation of Rubisco under HS might be a regulated response to a limitation elsewhere in the photosynthetic apparatus (Sharkey, 2005; Sage et al., 2008). As discussed above, the demand for de novo biosynthesized saturated FAs for the remodeling of membrane lipids during the first hours of HS potentially represents a significant sink for ATP and NADPH. Perhaps it is for coping with this new sink arising early during HS that cells suppress carbon fixation? A fast remodeling of membrane lipids after the onset of HS appears essential for survival, as plants with more saturated FAs in thylakoid membrane lipids were much more heat tolerant (Murakami et al., 2000). Also, the observation that the oxygen evolution capacity was reduced by two-thirds after 24 h HS while it remained unchanged during the first 3 h of HS might indicate that strong sinks for NADPH and ATP prevailed during the first 3 h but became much smaller between 3 and 24 h of HS (Figure 3H; Supplemental Table 1). While this would indicate long-term acclimation of LEF capacity according to reduced demands for NADPH and ATP, we cannot rule out damage in thylakoid membrane complexes as cause, potentially indicated by the accumulation of PLB-like structures.

The following observations suggest that carbon fixation by the Calvin cycle has to some extent resumed after 24 h of HS: (1) Algalized after 24 h of HS (Supplemental Data Set 2). (2) A putative activase from thermal denaturation (Salvucci, 2008) and that in *Chlamydomonas* partly resume between 3 and 24 h of HS, cells in this period would face an increased accumulation of H$_2$O$_2$. This idea appears supported by the upregulation after 24 h HS of several enzymes involved in the detoxification of H$_2$O$_2$ (Figures 5B and 8; Supplemental Data Set 2, Note 1). Among these are four hybrid cluster proteins (HCPs) that all contain putative organellar targeting signals and are upregulated 1.8- to 8.6-fold. An HCP ortholog in *E. coli* was known to be specifically induced by H$_2$O$_2$, and to confer protection toward hydrogen peroxide (Almeida et al., 2006). Moreover, three peroxiredoxins, PRX1, PRX2, and PRX4, targeted to chloroplast, cytosol, and mitochondria, respectively (Dayer et al., 2008), were upregulated 1.2-fold. Also, thioredoxin TRXh1, and ferredoxin-thioredoxin reductase (FRR2), most likely involved in the regeneration of oxidized peroxiredoxins in the respective compartments (Diez et al., 2006), were upregulated 1.2- to 1.9-fold after 24 h of HS. An increased demand under long-term HS for detoxifying H$_2$O$_2$ is also indicated by the 1.2- to 4.4-fold upregulation of several enzymes involved in the biosynthesis of glutathione (GSH1, a putative glutaryl-cysteine ligase), ascorbate (NED4, a putative sugar nucleotide epimerase), and the reduction of dehydroascorbate by glutathione (DAR1, a putative glutathione-dependent dehydroascorbate reductase; PD12, PD14, and RB60, protein disulfide isomerases also known to catalyze this reaction; Wells et al., 1990). An increased production of glutathione between 3 and 24 h of HS appears supported by the decline in abundances of cysteine and glutamate in this time period (Supplemental Figure 3B and Supplemental Data Set 1). That the abundance of glycine, the third amino acid forming glutathione, is increasing might be explained by its increased production by photorespiration.

However, the 1.4- to 1.9-fold upregulation of superoxide dismutases FSD1, MSD1, and MSD2, targeted to chloroplast, cytosol, and mitochondria, respectively (Page et al., 2012), also suggests the increased generation of superoxide (O$_2^\cdot$) under long-term HS. As the dismutases convert superoxide to H$_2$O$_2$, another source for the obviously increased accumulation of H$_2$O$_2$ might also be superoxide generated, e.g., during photosynthetic LEF by the Mehler reaction at PSI or in the mitochondrial respiratory chain at complexes I and III (Mehler, 1951; Turner, 1997; Asada, 2006). Either source appears surprising, as the capacities for LEF and respiration...
are reduced by two-thirds and half, respectively, between 3 and 24 h HS. However, the absence of sufficient sinks for electrons in the chloroplast may lead to the overreduction of ferredoxin and, thus, increased Mehler reactions (Schreiber and Neubauer, 1990). This scenario appears supported by the finding that the Chlamydomonas pgd1 mutant, impaired in TAG biogenesis, loses viability during N starvation as a consequence of ROS accumulation. In the absence of TAG biogenesis as sink for NADPH and ATP, ROS was suggested to derive from increased Mehler reactions (Li et al., 2012). Moreover, proper electron transfer in mitochondrial and plastidic membrane protein complexes might also be impaired because essential structural lipids PG and SQDG are depleted. While abundances of proteins involved in ROS scavenging did not change or even decreased during the first 3 h of HS (Mühlhaus et al., 2011), their accumulation after 24 h of HS can be clearly considered as a late response to HS. This also is in line with previous proteomics studies on different land plant species exposed to HS for more than 6 h, where levels of proteins like superoxide dismutase, thioredoxin h, ascorbate peroxidase, and dehydroascorbate reductase have been found to increase (Ferreira et al., 2006; Lee et al., 2007; Xu and Huang, 2008; Scafaro et al., 2010).

**During Recovery from HS, Cells Appear to Readjust Membrane Fluidity by Recycling Polysaturated FAs from Lipid Bodies into Membrane Lipids**

A first phase of recovery from HS, initiated by shifting cells back from 42 to 25°C, lasts for about 8 h and ends when cells start dividing again (Figures 2A and 8). Not surprisingly, during this time most measures taken by the cells during HS are reversed. As expected from the concept of homeoviscous adaptation (Sinensky, 1974), cells adapt membrane fluidity again to cooler temperatures and do so by increasing the content of polysaturated FAs at the expense of saturated ones in membrane lipids (Figure 7; Supplemental Figure 5). Since in TAGs at the same time the content of polysaturated FAs decreased while that of more saturated FAs increased, it appears that once again an exchange of FAs between TAGs and membrane lipids occurred (Figure 6; Supplemental Figure 5). This seems surprising because desaturation of FAs in membrane lipids can be achieved rapidly by desaturases (Russell, 1984). The answer might be associated with FA chain length; as shown in Figures 7B to 7D, FAs in accumulating lipids not only are more unsaturated, they are also longer, while those in declining lipids not only are more saturated but also shorter. Hence, desaturation would need to come along with chain elongation. In support of the idea that unsaturated, long-chain FAs are recycled from lipid bodies rather than biosynthesized de novo, enzymes BCC1 and FAB2, involved in elongation and desaturation of FAs of plastidic lipids, are downregulated by ∼20% during recovery (Supplemental Data Set 2).

**Recovery from HS Is Characterized by the Depletion of Stress Protectants and the Resumption of Anabolic Reactions**

Apparent, TCA/glyoxylate cycle, glycolysis/gluconeogenesis, and the Calvin cycle increased their activities during recovery, as judged from the accumulation of succinate, fumarate, 2-oxoglutarate, citrate, malate, glycercate, glycercate-3-phosphate, and starch (Figures 2E and 4; Supplemental Figure 3 and Supplemental Data Set 1). By contrast, levels of GPG, trehalose, and amino acids asparagine, isoleucine, alanine, and threonine declined rapidly during recovery, qualifying them as potential compatible solutes. This is because compatible solutes, protecting denatured proteins from aggregation during HS, need to be degraded during recovery as they would inhibit the reactivation of denatured proteins by molecular chaperones (Singer and Lindquist, 1998a, 1998b). As bulk protein increased by only 10% during the 8 h of recovery (Figure 2D), the decline of amino acids probably only to some extent is due to protein biosynthesis. Rather, they might be utilized for the biosynthesis of purines and pyrimidines for DNA replication. Also, the degradation of trehalose and GPG may rapidly provide ribose sugars and phosphate for DNA biosynthesis.

**Resumption of Cell Division Lags Behind Resumption of DNA Replication**

DNA biosynthesis resumed 2 to 3 h after shifting cells back to 25°C and came along with the depletion of guanosine (Figure 2B; Supplemental Figure 3B). At this time, cell cycle arrest in G1 apparently is relieved and cells enter the S1 state. However, it is not clear why the ∼14% of 2n cells in the population of heat-stressed cells do not directly enter mitosis nor why the rapidly increasing number of 2n cells during recovery do not. As such retarded G2/M progression was reported for the Chlamydomonas fa2 mutant, lacking a NIMA kinase (Mahjoub et al., 2002), perhaps NIMA kinase members are thermolabile and regain activity only late during recovery? Surprisingly, despite a rapid onset of DNA biosynthesis, the biosynthesis of most histone proteins lagged behind, such that the total cellular histone content was reduced by ∼26% after 8 h recovery compared with pre-HS conditions (Figure 5B; Supplemental Data Set 2, Note 1). This suggests the existence of a nonassembled pool of histones in Chlamydomonas, as reported earlier in mammalian cells (Tsvetkov et al., 1989), perhaps to funnel protein biosynthesis capacity to more important targets. One such target might be ribosomal proteins, whose levels increase during the 8-h recovery period by more than they have decreased during the 24-h HS period, leading to ∼10% more ribosomes after 8 h recovery compared with pre-HS conditions (Figure 5B; Supplemental Data Set 2, Note 1).

**Readjustment of Protein Abundance during Recovery Often Is Selective**

During the 8-h recovery period, 38 proteins decreased significantly more than expected from dilution by the 10% increase in bulk protein in this period and therefore must have higher degradation than biosynthesis rates (Supplemental Data Set 2, Note 2). Interestingly, 13 of these proteins are molecular chaperones and immunophilins, thus indicating that an excess of molecular chaperones is not beneficial under nonstress conditions. This might be because of futile ATP hydrolysis by the HSP100, HSP90, HSP70, and HSP60 chaperones or because “overchaperoning” is counterproductive for assisting folding of denatured proteins to the native state (Bersuker et al., 2013). Even more interesting is that
HSP100 family member ClpD1 and chloroplast HSP70B cocha-
perone CGE1 increased even further during recovery. For ClpD, this
exceptional behavior is difficult to explain as a clear functional
assignment for this protein is yet lacking (Sjögren et al., 2014).
During long-term HS, levels of HSP70B increased 2.6-fold, while
those of CGE1 only by 1.6-fold (Supplemental Data Set 2). As
CGE1 accelerates nucleotide exchange, the increased HSP70B to
CGE1 ratio during HS may increasingly shift HSP70B toward the
ADP-bound state and, thus, from a folding-supportive to a holdase
activity (Veyel et al., 2014b). Hence, decreasing HSP70B and in-
creasing CGE1 levels during recovery may rapidly shift the system
back to a folding-supportive function.

For proteins involved in photosynthesis, we observed a similar
reversal of changes that took place during HS. Regarding the
light reactions, this is evidenced by a mild −6% increase in
ATPase subunits and a decrease of PSII subunits (Figure 8;
Supplemental Data Set 2, Note 1). Most of the latter do not
decrease by significantly more than 10%, resulting from dilution
by bulk protein increase during recovery and thus may not be
actively degraded. The overall modest adjustments of light re-
action proteins during recovery are accompanied by similarly
modest relaxations of LHC uncoupling, increases in PSII quan-
tum yield and maximum quantum efficiency, and chlorophyll a/b
ratio (Figure 3; Supplemental Table 1). Accordingly, PLB-like
structures disappear by about half only between 4 and 8 h of
recovery (Supplemental Figure 2).

The imbalance between the 30 to 40% decrease in Rubisco
and −60% increase in Rubisco activase generated during long-
term HS is corrected during recovery such that Rubisco reaches
80% and Rubisco activase 100% of initial levels. These read-
justments most likely increase Calvin cycle activity to meet the
need of ribose for DNA biosynthesis. Although levels of PGP1
decreased by 19% during recovery, they were still 1.9-fold
higher after 8 h recovery compared with pre-HS levels. The new
sink for ATP and NADPH provided by DNA biosynthesis should
decrease ROS that was produced as consequence of an over-
reduced photosynthetic electron transport chain (Schreiber and
Neubauer, 1990; Li et al., 2012). Accordingly, almost all proteins
involved in ROS scavenging declined during recovery, with the
exception of TRXo, HCP2, and HCP3, which continued to in-
crease. The known or predicted mitochondrial localization of
these three proteins suggests increased ROS production by
mitochondrial respiration during recovery.

The continued increase of enzymes involved in BCAA bio-
synthesis during recovery is surprising, as levels of isoleucine
and leucine declined (Figure 4A; Supplemental Figure 3). Cells
might attempt to counteract the consumption of BCAA during
recovery; as shown previously for heat-stressed Salmonella
typhimurium, BCAAs for unknown reasons improve recovery
from HS (Hsu-Ming et al., 2012).

A Modified Growth Phase Is Indicated by the Resumption of
Cell Division Despite Remodeled Protein, Metabolite, and
Lipid States

After −8 h of recovery from HS Chlamydomonas cells regained
their ability to divide (Figures 2A and 8). This appears surprising
because at this time the composition of metabolites, membrane
lipids, and proteins was clearly distinct from pre-HS conditions
(Figures 4B, 5A, and 7E). Specifically, levels of several meta-
obolites of the central metabolism were significantly higher after 8
h of recovery compared with pre-HS conditions (Supplemental
Data Set 1 and Supplemental Figure 3). Moreover, DGTS spe-
cies with polyunsaturated long-chain FAs were strongly re-
duced, as were most SQDG species, while MGDG, DGDG, and
DGTS species with short, largely saturated FAs were enriched
(Supplemental Figure 5). While proteins of functional categories
protein folding, abiotic (heat) stress, redox, and BCAA synthesis
were still upregulated and histones still downregulated after 8 h
recovery compared with pre-HS conditions, ribosomal proteins
had overaccumulated (Figure 5B). This might have been trig-
gered by the overaccumulation of starch, TAGs, and central
metabolites potentially signaling ample resources for protein
biosynthesis upon relief of cell cycle arrest. Proteins involved
in photosynthesis also had not relaxed to the pre-HS state. For
example, subunits of PSII remained increased by −20% above,
and those of the ATP synthase remained decreased by −12%
below initial levels (Supplemental Data Set 2, Note 1). Accord-
ingly, and potentially exacerbated by the lasting deficiency of
thylakoid membrane lipid SQDG, LHC uncoupling, reduced PSII
quantum yield/maximum quantum efficiency, and reduced chlorophyll a/b ratio prevailed, as did aberrant PLB-like struc-
tures in thylakoid membranes (Figures 2F and 3; Supplemental
Figure 2).

Overall, it appears that Chlamydomonas cells during recovery
focus on processes enabling them to resume cell division and
growth as quickly as possible (DNA biosynthesis, overaccumula-
tion of central metabolites, TAGs, starch, and ribosomes) rather
than the restoration of a cellular state apparently optimal for growth at
lower temperatures (e.g., original compositions of membrane
lipids and chaperones; full antenna coupling). This strategy
might be based on the fact that dilution by growth will eventually
restore the balance of cellular compounds; the availability of
ample carbon storage may abolish the need to fully restore
photosynthesis. However, as these “imbalances of cellular
compounds” in fact are the result of acclimation to HS, cells may
also maintain them in the frame of a precautionary concept to be
prepared for another HS. Perhaps in support for the latter idea,
average cell diameter and average contents of DNA, protein,
and chlorophyll were higher after 24 h of recovery compared with
pre-HS conditions, although cells had divided already −1.6
times (Figure 2). Experiments monitoring cellular states for
days after recovery from prolonged HS will be required to
test whether such a “stress memory,” potentially realized by
epigenetic marks (Thellier and Lüttge, 2013), might exist in
Chlamydomonas.

HS Represents Another Trigger for TAG Accumulation with
Potential Impact on Biodiesel Production

Oleaginous algae produce only small quantities of TAGs under
optimal growth conditions but accumulate large amounts of
TAGs when placed under stress conditions like nutrient starva-
tion, salinity, changes in medium pH, temperature, or light in-
tensity (Hu et al., 2008). Although Chlamydomonas was not
considered to be an oleaginous alga (Sheehan, 1998), depriving
it from N or S results in the accumulation of considerable amounts of TAGs in lipid bodies (Wang et al., 2009; Cakmak et al., 2012). The results from our study suggest that heat stress can be considered as another stress causing TAG accumulation potentially exploitable for biodiesel production with Chlamydomonas. As heat stress and N and S starvation lead to a cell cycle arrest, the latter might be a general trigger for TAG accumulation, as suggested earlier (Sheehan, 1998).

The suitability of algal biodiesel producers is not only determined by the quantity of TAGs but also by the degree of FA saturation, as more saturated FAs shorten ignition delay time and ensure better combustion quality (Knothe et al., 1998; James et al., 2013). In this respect, we observed a rapid increase of TAGs with highly unsaturated FAs within the first 2 h of HS, which during prolonged HS and especially during recovery became more saturated (Figures 6 and 8). Hence, high-quality biodiesel may be produced by exposing Chlamydomonas cells to prolonged HS at 42°C followed by recovery at lower temperatures. Such conditions are found in hot desert climates during the day/night cycle and therefore may minimize costs for heating and chilling. In this study, we supplemented growth medium with acetate. Hence, it is important to test whether TAG accumulation during HS also takes place under photoautotrophic growth conditions. As this is the case for N-starved Chlamydomonas cells (Merchant et al., 2012), it is likely to be true also under HS conditions.

Can Data on Chlamydomonas Be Used to Improve Thermotolerance in Crop Plants?

Our system-wide and time-resolved analysis of acclimation responses of Chlamydomonas to prolonged HS suggests a temporally ordered, orchestrated implementation of response elements at various system levels (Figure 8). Hence, the best way to improve crop plant thermotolerance probably would be to trigger the entire cascade of responses before an expected heat wave, ideally by applying a degradable compound. However, this requires detailed knowledge on HS sensing and involved HS signaling pathways. A sufficient increase in thermotolerance may also be achieved by the triggering of single response elements, like the generation of compounds involved in stress protection from the catabolism of larger molecules or the biosynthesis of membrane lipids with more saturated FAs. This may be achieved rather easily by engineering involved key enzymes toward an externally controllable activity. However, we first need to know the contributions of individual HS response elements to overall thermotolerance. Moreover, it is possible that a response element implemented in isolation would have negative effects. Clearly, more research on the plant HS response is required to address this issue.

METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii strain CF185 (CF185, cw9, mt+, arg7), complemented with plasmid-derived ARG7 wild-type gene (Schroda et al., 1999) was used in all experiments. Cells were grown mixotrophically in Tris-acetate-phosphate (TAP) medium on an orbital shaker at 120 rpm, 25°C, and constant illumination at ~40 μE m⁻² s⁻¹. Cells were counted and mean cell diameter was determined using a 22 Coulter Counter (Beckman Coulter).

For the HS recovery time course, CF185 cells were grown at constant illumination in TAP medium to mid-log phase, harvested by centrifugation at 25°C and 1950g for 4 min, resuspended in TAP medium prewarmed to 42°C, incubated at 42°C in an agitating water bath for 24 h at constant illumination (~40 μE m⁻² s⁻¹), and transferred back to pre-HS conditions.

DNA Content and Ploidy

Prior to DNA staining, cells were destained and fixed with ethanol (Garz et al., 2012). Fixed cells were washed two times with ice-cold PBS buffer and were kept in PBS at 4°C. DNA in cells was stained with propidium diiodide (PI) (Sigma-Aldrich), dissolved in PBS (final concentration 2.5 μg/mL suspension; Coulson et al., 1977; Coulson and Tyndall, 1978), and simultaneously treated with RNase A (final concentration 10 μg/mL) for 3 h at 4°C. Prior to cytometric analysis, cells were washed once with ice-cold PBS, RNase A and PI solution were freshly prepared before every experiment. To avoid bleaching, PI-treated cells were kept in the dark until the analysis was finished. PI-dependent fluorescence was quantified by flow cytometry using a FACScalibur instrument (Becton Dickinson). For excitation, a 488-nm argon laser was used. PI-derived fluorescence was passed through a band-pass filter (central wavelength, 585 nm; half bandwidth, 42 nm). Fifty thousand cells were analyzed for each sample. Count rates did not exceed 4000 counts per second. Data were collected with CellQuest software (Becton Dickinson) and analyzed by a program written in MATLAB (The MathWorks).

Chlorophyll Content

Cell culture samples (200 μL) were snap frozen in liquid nitrogen and stored at −80°C. Acetone was added to thawed samples to a final concentration of 80%. Cells were ruptured mechanically and centrifuged to remove cells debris. Chlorophyll absorption of supernatants was measured photospectrometrically (Lambda 25, UV/VIS spectrometer; Perkin-Elmer) at 646.6 and 663.6 nm and corrected against the absorption at 750 nm (chlorophyll a/b = 0.01776 × E₆₄₆.₆ + 0.00734 × E₆₆₃.₆) (Porra et al., 1989).

Total Protein Content

Cell culture samples (8 mL) were harvested at 4°C and 3220g for 2 min, the supernatant was discarded, and the pellet was frozen in liquid nitrogen and stored at ~80°C. The cell pellet was resuspended and incubated in 1 mL Lowry’s solution (solution A: 4 mg/mL NaOH + 20 mg/mL Na₂CO₃ in H₂O; solution B: 10 mg/mL potassium sodium tartrate + 5 mg/mL CuSO₄ in water; mix solution A + B at 50:1 ratio) for 15 min (Lowry et al., 1951). After adding 100 μL 1 × Folin’s reagent, the suspension was incubated for another 30 min. Samples were measured photospectrometrically (Lambda 25, UV/VIS spectrometer) at 750 nm.

Starch Content

The starch-containing fractions of polar and semipolar metabolites gained during lipophilic metabolite extraction were dried in a speed vac concentrator, reconstituted in 400 μL 0.1 M sodium hydroxide (NaOH), and boiled for 30 min at 95°C. To neutralize the solution, 80 μL HCl-acetate buffer was added (0.5 M HCl and 0.1 M acetate/NaOH, pH 4.9). Each sample (240 μL) was mixed in a 1:1 ratio with starch assay reagent (SA-20 kit; Sigma-Aldrich) and incubated overnight at 37°C on an orbital shaker. Afterwards, samples were centrifuged at 25°C and 1100g for 5 min, 50 μL of each sample was dispensed in a microtiter plate, and 150 μL glucose assay reagent (SA-20 kit; Sigma-Aldrich) added. The mixture was incubated for 15 min at 25°C and the absorbance measured at 340 nm. Controls and calculations were performed according to the manufacturer’s instructions (SA-20 kit).
Electron Microscopy

Cell culture samples (8 mL) were harvested at room temperature and 1000g for 2 min. The supernatant was discarded, the pellet was washed with 100 mM sodium cacodylate (pH 7.2) and centrifuged at 1500g for 1 min, and cells fixed for 2 to 14 h at 25°C in 100 mM sodium cacodylate containing 2.5% glutaraldehyde (pH 7.2). Samples were processed further as described by Nordhues et al. (2012).

Spectroscopy

The 77K fluorescence emission spectra were recorded using a Jasco F-6500 fluorimeter. Two replicates of whole cells from the culture flasks (~5 μg/mL total chlorophyll) were frozen directly in liquid nitrogen. Chlorophyll fluorescence was excited at 440 nm and fluorescence emission was recorded from 655 to 800 nm. Data were corrected for the average fluorescence emission between 790 and 800 nm and normalized either for the PSI emission maximum around 687 nm or for the PSI emission maximum around 712 nm.

Light response curves of chlorophyll fluorescence at room temperature were recorded using a Dual PAM 100 pulse amplitude-modulated fluorimeter (Heinz Walz). Before measurements, the cells were illuminated for 5 min with far-red light followed by 5 min of dark adaption. Each light response curve of chlorophyll fluorescence was fitted using the model of Eilers and Peeters (1988) integrated in the DualPAM v1.11 software (Heinz Walz). Resulting parameters were calculated.

Photosynthesis-Irradiation Curves/Oxygen Evolution and Consumption

Oxygen evolution and consumption were determined from three biological and three technical replicates each. Cells were taken prior to, 3 h, 10 min to 25°C, and NaHCO3 was added to a final concentration of 10 mM. A 1-mL sample of this suspension was placed into a multiport measuring chamber (NOCHM-4; World Precision Instruments). The temperature-controlled multiport measuring chamber was connected to an optical oxygen measurement system (oxy-4 mini; PreSens) and illuminated by a surrounding LED ring. Light intensities within cell suspensions were determined by Spherical Micro Quantum Sensor US-500/L connected to a DUAL-PAM-100 (Walz). Oxygen evolution was monitored for 4 min at different light intensities (50, 150, and 600 μM m⁻² s⁻¹), whereas oxygen consumption was recorded in the dark. Assuming that oxygen solubility in water at 25°C under atmospheric pressure is 258 μM, oxygen evolution and consumption were calculated based on time and cellular chlorophyll content (μmol O2 h⁻¹ mg chlorophyll⁻¹).

¹⁵N Quantitative Shotgun Proteomics

Cells were harvested by centrifugation at 4°C and 3220g for 2 min, washed with 5 mM HEPES-KOH, transferred to Eppendorf tubes, centrifuged again at 4°C and 21,500g for 2 min, resuspended in lysis buffer (50 mM NH₄HCO₃, 1 mM DTT, and 1 mM NaCl), frozen in liquid nitrogen, and stored at −80°C. ¹⁵N-labeled reference cells, grown and harvested as described (Mühlhaus et al., 2011), were mixed with non-labeled samples from the HS and recovery time course at a ¹⁵N/¹⁴N ratio of 0.8 based on protein content determined by the BCA assay (Thermo Scientific). Mixed cells were disrupted by two freeze/thawing cycles, and soluble and membrane-associated proteins were separated by centrifugation in a table lab centrifuge at 4°C and 21,500g for 35 min. The supernatant, containing soluble proteins, was recovered and the pellet, containing membrane associated proteins, was washed once in lysis buffer (50 mM NH₄HCO₃, 1 mM DTT, and 1 mM NaCl) and centrifuged again. The resulting pellet, including the membrane-associated proteins, was again resuspended in lysis buffer. Samples containing 200 μg soluble and membrane-associated proteins as determined by the BCA assay were precipitated in 80% acetone over night at −80°C. Precipitated proteins were resuspended in 6 M urea, 2 M thiourea, reduced with 0.24 mM DTT at 25°C for 30 min, and carbamidomethylated with 1 mM iodoacetamide at 25°C for 20 min in the dark. Samples were diluted with 40 mM ammonium hydrogen carbonate to 3 M urea and 1 M thiourea and digested with 0.5 μg endoprotease Lys C (Roche) on a rotating wheel for at least 3 h at 37°C. The digest was diluted to 1.5 M urea and 0.5 M thiourea with 20 mM ammonium hydrogen carbonate and 5% acetonitrile and digested with 10 μL immobilized trypsin (Poroszyme immobilized trypsin; Applied Biosystems) for at least 16 h at 37°C on a rotating wheel. The resulting peptide composite was diluted with LC-MS-grade water to a final acetonitrile concentration of 2.5%, desalted using SPEC-C18 solid phase extraction plates (Varian/Agilent Technologies), and dried in a centrifugal evaporator. The dried peptides were resuspended in 2% acetonitrile and acidified with 0.5% acetic acid.

Liquid chromatography and mass spectrometry of peptides were performed according to Mühlhaus et al. (2011) with the exception that the MS spectra were acquired at a resolution of 60,000 and the six most intense precursor ions were selected for MS2 analysis. All single charged ions were excluded from the analysis in two of three technical replicates of each sample and dynamic exclusion was set 30 s after two occurrences. Proteins were identified and quantified using the IOMIQS framework (Mühlhaus et al., 2011). IOMIQS features four different search engines for peptide identification: Mascot (version 2.2.04; Perkins et al., 1999), Sequest (version 28; Eng et al., 1994), OMSSA (version 2.1.4; Geer et al., 2004), and XTandem (version 2009.04.01.1; Craig and Beavis, 2004). For peptide quantification, IOMIQS employs XPRESS (Han et al., 2001).

For database searches, the precursor mass tolerance was set to 10 ppm (for OMSSA, it was set to 0.015 D), whereas the fragment ion tolerance was set to 0.8 D. Up to three missed cleavages were allowed for tryptic peptides. As variable modifications, carbamidomethylation of cysteine and oxidation of methionine were selected for Mascot, OMSSA, and XTandem. To identify ¹⁵N-labeled peptides, the searches were repeated with exactly the same settings but in ¹⁴N mode. The database search was performed against a combined protein sequence database including all translated sequences of Chlamydomonas Augustus gene models version 10.2 as well as all sequences from Chlamydomonas reinhardtii and chloroplast proteins (downloaded from http://chlamycyc.mpimp-golm.mpg.de/files/sequences/protein/). In addition, all target sequences were shuffled and added to the search database to perform the target decay approach for FDR calculation (Elias and Gygi, 2007). The FDR distributions of all search algorithms were aligned to allow for overall peptide identifications with greater than 95% probability. For protein inference, peptides were classified according to their information content according to Qeli and Ahrens (2010). The Occam’s Razor approach (Nesvizhskii et al., 2003) was applied to each peptide class from high to low information content to report a minimal set of proteins explaining the identified peptides by proteins and protein groups. Proteins were classified according to their peptide(s) with the maximal information content.

For peptide quantification, XPRESS was used with peptide mass tolerance set to 0.8 D. The “light-to-heavy” ratios calculated by XPRESS for identified peptides were corrected for unequal mixing of labeled and unlabeled proteins in each sample by the geometric median of light-to-heavy ratios of all peptides in that sample. The median of peptides light-to-heavy ratios was used to determine the relative abundance for the corresponding proteins. The mean and so was calculated from three biological and three technical replicates. For protein quantification, both fractions (soluble and membrane-associated proteins) were pooled and mean and so was calculated from three biological and three technical
replicates each. Proteins that showed significant pairwise anticorrelation between the two fractions were excluded from the data set.

**GC-MS: Polar Metabolites**

The experiments were done in three independent biological replicates and from each culture four sample replicates were harvested and processed. For profiling polar metabolites, 2.5 to 3 mL cell culture was harvested by fast filtration as described by Krall et al. (2009), which took around 15 to 30 s. The frozen filters with cells were stored at −80°C until extraction. Cells were extracted using a mixture of methanol and chloroform (7:3, v:v) including internal standards (0.02 mg/mL 13C6-sorbitol and 0.2 mg/mL d4-alanine). Extraction buffer (300 µL) precooled to −20°C was added to the frozen sample, followed by vortexing for 10 min at 4°C. The sample was quickly spun down and the liquid was transferred into a new tube. Another 300 µL extraction buffer was used to wash the filter and added to the first 300 µL. The mixture was then strongly vortexed at 4°C for 60 min. Ultrapure water (300 µL: LC-grade; Merck) was added to the extraction mixture for phase separation into methanol-water and chloroform phase. To break any remaining intact cells, the sample was twice frozen in liquid nitrogen and thawed on ice. The sample was centrifuged at 21,500g, 0°C for 10 min, and 700 µL methanol-water phase was transferred to a new tube, dried in a speed vac overnight, flushed with Argon, and stored at −80°C.

Sample derivatization and GC-MS analysis of polar metabolites was done as described previously (Strehmel et al., 2010; Mettler et al., 2014), except that during derivatization only 20 and 40 µL of volume for the methoxamination and syillation steps were used, respectively. The ChromaTOF software (version 3.22; LECO) was used to visually inspect, baseline correct, and export the GC-MS raw data into NetCDF file format with the following settings: baseline offset, 1.0; smoothing of data points, 5; signal-to-noise ratio, 10. Further preprocessing, including peak finding, data matrix construction, and analyte annotation, was done using the TagFinder software (Luedemann et al., 2008) using the spectral reference library of the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/; Kopka et al., 2005; Schauer et al., 2005; Hummel et al., 2010), dated 3/15/2011. At least four matching masses were required with a minimum match value of 750 and a maximum retention index deviation of 1.0 for the positive annotation of an analyte (Strehmel et al., 2008). From the annotated analytes, a data profile was calculated, where the intensities of each analyte were scaled to 100 over the different samples. The generated data matrix was normalized by the internal standard 13C5-sorbitol, the sampled biomass, followed by probabilistic quotient normalization (Dieterle et al., 2006) with the average from all chromatograms set as reference chromatogram. Next, standards were removed from the data (normalized GC-MS raw data; Supplemental Data Set 1). Values of analytes representing the same metabolite were summed and technical replicates were averaged prior to further analysis.

**LC-MS: Lipophilic Metabolites**

The experiments were done in three independent biological replicates and from each culture two sample replicates were harvested and processed. Samples (2 mL) of cell culture were harvested by centrifugation at 4°C and 21,500g for 2 min. The supernatant was removed and the cell pellet was directly frozen in liquid nitrogen and stored at −80°C. Extraction of lipophilic metabolites and LC-MS measurements was done as described by Hummel et al. (2011). Known exact masses were used to extract data of 198 lipids from the obtained chromatograms (Hummel et al., 2011). The resulting data matrix comprised 196 metabolites and initially 48 samples (columns) of which two samples were removed prior to further analysis. The data was normalized to the internal standard PC 34:2 and background (average of 8 blank samples) was subtracted. The resulting data were subjected to probabilistic quotient normalization (Dieterle et al., 2008) with the average of all chromatograms set as reference chromatogram (normalized LC-MS raw data; Supplemental Data Set 4). The normalized data were averaged over the technical replicates and used for further analyses. Heat maps were generated with the gplots-package in R (http://www.r-project.org/) and reworked in Adobe Illustrator (Adobe Systems).

**General Analysis of Molecular Data**

All samples were normalized method specific to minimize technical influences. For assessing significant changes of metabolites and proteins, data were log2 transformed and tested with a one-way ANOVA over all time points using a significance threshold (P value < 0.05) after correction for multiple hypothesis testing according to Benjamini and Hochberg (1995). To investigate common behaviors in the data sets, hierarchical clustering was performed. For this, polar metabolites and protein data were log2 transformed and lipid and TAG data z-transformed [Z = (X − μ)/σ, where μ = mean of X and σ = SD of X]. The hierarchical clustering was performed using Euclidian distance measure and Ward linkage criterion.

Sample derivatization and GC-MS analysis of polar metabolites was done as described previously (Strehmel et al., 2010; Mettler et al., 2014), except that during derivatization only 20 and 40 µL of volume for the methoxamination and syillation steps were used, respectively. The ChromaTOF software (version 3.22; LECO) was used to visually inspect, baseline correct, and export the GC-MS raw data into NetCDF file format with the following settings: baseline offset, 1.0; smoothing of data points, 5; signal-to-noise ratio, 10. Further preprocessing, including peak finding, data matrix construction, and analyte annotation, was done using the TagFinder software (Luedemann et al., 2008) using the spectral reference library of the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/; Kopka et al., 2005; Schauer et al., 2005; Hummel et al., 2010), dated 3/15/2011. At least four matching masses were required with a minimum match value of 750 and a maximum retention index deviation of 1.0 for the positive annotation of an analyte (Strehmel et al., 2008). From the annotated analytes, a data profile was calculated, where the intensities of each analyte were scaled to 100 over the different samples. The generated data matrix was normalized by the internal standard 13C5-sorbitol, the sampled biomass, followed by probabilistic quotient normalization (Dieterle et al., 2006) with the average from all chromatograms set as reference chromatogram. Next, standards were removed from the data (normalized GC-MS raw data; Supplemental Data Set 1). Values of analytes representing the same metabolite were summed and technical replicates were averaged prior to further analysis.

**Functional Enrichment of Proteins**

Identified proteins were functionally annotated using MapMan (Thimm et al., 2004) for *Chlamydomonas* including changes made by the community within the IOMIQS functional annotation tool (T. Mühlhaus, A. Lüdemann, and M. Schroda, unpublished data). The analysis of over-representation of certain functional categories was done using hyper-geometric formulation of the null hypothesis to test that the change in protein abundance and its belonging to an annotation category is statistically independent (Rivals et al., 2007). P values were adjusted according to the Benjamini and Hochberg procedure and the significance threshold was set to a P value < 0.05. To calculate functional enrichment of proteins during heat stress and recovery the total protein population for which we have behavioral information (1985) in our experiment and the number of differentially changing proteins (772) were used. In addition, overrepresentation of functional categories on each comparison between time points with maximal variance was determined. The overlap in these global and local enrichment calculations was used to gain a more robust analysis. The hierarchical topology of the MapMan annotation was simplified using only upper parent category for visualization.

**Comparison of BCAA Content per Protein**

To compare the BCAA content in different protein sets, quantile distributions of BCAA per protein were calculated for each set (Karlin and Brendel, 1992). The significance of the differences of the distributions was assessed by a two-tailed Student’s t test (Baudouin-Cornu et al., 2001). The protein sets were defined by the contrast between time point 0 and 24 h HS, also assessed by two-tailed Student’s t test, and divided into
up- and downregulated proteins. The cellular proteome based on Augustus 10.2 gene models was used as reference set.

**Determination of Minimal Set of Actively Degraded Proteins**

To separate the set of proteins decreasing in abundance between two time points (T₁ and T₂) into subsets of proteins (A) actively degraded and (B) diluted by the increase of bulk protein, the expected dilutions at each contrast (T₂ versus T₁) were calculated (Equation 1).

\[
\text{Expected dilution} \% = \left( \frac{\text{protein}_A}{\text{protein}_B} - 1 \right) \times 100\%
\]

Individual proteins whose abundance was below that expected from dilution were assigned to set A and all others to set B. The statistical significance was determined by a two-tailed Student’s t test followed by Benjamini and Hochberg correction. The significance threshold was set to a P value ≤ 0.01 for a stringent selection of actively degraded proteins.

**Accession Numbers**

Sequences of the genes/proteins mentioned in this work can be found at Phytozome (http://www.phytozome.net/) using the gene identifiers provided in Supplemental Data Set 2 or via the Functional Annotation tool (http://iomiqsweb1.bio.uni-kl.de/).

**Supplemental Data**

The following materials are available in the online version of this article.

- Supplemental Figure 1. Starch Quantification via Planimetry.
- Supplemental Figure 2. Development of Prolamellar Body-Like Structures during Heat Stress and Recovery.
- Supplemental Figure 3. Behavior of All 43 Significantly Changing Metabolites and Corresponding Enzymes in Cellular Metabolic Pathways.
- Supplemental Figure 4. Protein Cluster Tree.
- Supplemental Figure 5. Heat Map Illustrating the Behavior of All Measured Triacylglycerol and Lipid Species during the HS and Recovery Time Course.
- Supplemental Figure 6. TAG Cluster Tree.
- Supplemental Figure 7. Comparison of Proteome States at 3 h HS and 24 h HS.
- Supplemental Figure 8. Comparison of Branched-Chain Amino Acid Content between Proteins Up- and Downregulated after 24 h HS.
- Supplemental Table 1. Photosynthesis Parameters.
- Supplemental Data Set 1. Polar Metabolite Data.
- Supplemental Data Set 2. Protein Data.
- Supplemental Data Set 3. Protein Cluster Enrichment.
- Supplemental Data Set 4. Lipophilic Metabolite Data.

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**AUTHOR CONTRIBUTIONS**


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Systems-Wide Analysis of Acclimation Responses to Long-Term Heat Stress and Recovery in the Photosynthetic Model Organism Chlamydomonas reinhardtii

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