Systemic Cold Stress Adaptation of Chlamydomonas reinhardtii*

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*Chlamydomonas reinhardtii is one of the most important model organisms nowadays phylogenetically situated between higher plants and animals (Merchant et al. 2007). Stress adaptation of this unicellular model algae is in the focus because of its relevance to biomass and biofuel production. Here, we have studied cold stress adaptation of C. reinhardtii hitherto not described for this algae whereas intensively studied in higher plants. Toward this goal, high throughput mass spectrometry was employed to integrate proteome, metabolome, physiological and cell-morphological changes during a time-course from 0 to 120 h. These data were complemented with RT-qPCR for target genes involved in central metabolism, signaling, and lipid biosynthesis. Using this approach dynamics in central metabolism were linked to cold-stress dependent sugar and autophagy pathways as well as novel genes in C. reinhardtii such as CKIN1, CKIN2 and a hitherto functionally not annotated protein named CKIN3. Cold stress affected extensively the physiology and the organization of the cell. Gluconeogenesis and starch biosynthesis pathways are activated leading to a pronounced starch and sugar accumulation. Quantitative lipid profiles indicate a sharp decrease in the lipophilic fraction and an increase in polyunsaturated fatty acids suggesting this as a mechanism of maintaining membrane fluidity. The proteome is completely remodeled during cold stress: specific candidates of the ribosome and the spliceosome indicate altered biosynthesis and degradation of proteins important for adaptation to low temperatures. Specific proteasome degradation may be mediated by the observed cold-specific changes in the ubiquitinylation system. Sparse partial least squares regression analysis was applied for protein correlation network analysis using proteins as predictors and Fv/Fm, FW, total lipids, and starch as responses. We applied also Granger causality analysis and revealed correlations between proteins and metabolites otherwise not detectable. Twenty percent of the proteins responsive to cold are uncharacterized proteins. This presents a considerable resource for new discoveries in cold stress biology in algae and plants. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.026765, 2032–2047, 2013.

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Microalgae have a plastic metabolism allowing them to cope with many biotic and abiotic factors that may occur in its environment. Not so much is known about cold stress for algal metabolism, including chilling (below 15 °C) and freezing (below 0 °C). In Chlamydomonas, a moderate chilling stress lead to a decreased growth rate, chlorosis, progressive membrane and oxidative damage (1) whereas severe stress, with temperatures below 3 °C, lead to cell death after a short period. These injuries are similar to those observed in higher plants (2), in which growth and development are also reduced by a direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic, oxidative, energetic, and other stresses (3).

The chilling syndrome and its related injuries, in which no mechanical damage is produced as a consequence of ice crystals, can be explained both by structural membrane damage (alterations of membranes fluidity, breakage of cell structures) (4, 5) and decreased enzymatic kinetics as a consequence of protein denaturation resulting in a reduced or total loss of enzymatic activity. It was described that cold partially denatures the enzyme Thioredoxin h in Chlamydomonas, leading to a reduced activity (6). To avoid these effects that may affect all of the metabolic pathways and withstand low temperatures, the cells should accomplish numerous physiological, biochemical and molecular changes. This process, called acclimation, is not described in Chlamydomonas whereas extensively studied in higher plants (7, 8): Stress-related genes are induced (9, 10), antioxidative mechanisms (11), cryoprotectant osmolytes and proteins are increased (12, 13), lipid composition is altered and membranes are stabilized (14, 15) whereas photosynthesis (16), nutrient absorption, and growth are decreased (17).

The physiological changes listed above are the consequence of a major remodelling of the proteome, a complex process that involve not only stress sensing, signaling, and gene regulation, but also transcript and protein processing and degradation. In higher plants part of the sensing process starts with the change in the membrane fluidity, which at the end activates the cold responsive and ICE-CBF elements which regulate gene expression, also sharing some elements with drought response via drought responsive elements and MAPK cascades (for a review see (3, 18, 19)). A second signaling mechanism is the monitoring of the changes in the central metabolism. It has been proposed that energy-deficiency signals, specific sugars, triggers specific responses.
This mechanism is based on the enzymes hexokinase (HKX) and SnRK1 (SNF related kinase 1) (for a review on sugar signaling see (20–22). SnRK1 is inhibited by phosphate sugars, and under energy deficit it activates transcription factors, including bZIPs (23), general stress response elements, and histone modification leading to epigenetic changes. The activation of this enzyme leads to limiting cell growth and division, autophagy, protein, lipid, cell wall, and starch degradation, while promoting photosynthesis, gluconeogenesis, and sucrose metabolism. On the other hand HKX is associated to increased biosynthetic processes (24), also down-regulating SnRK1 by the production of hexoses-phosphate. Other sugars like trehalose and trehalose-phosphate, proposed to act downstream of SnRK1, are related to inhibitory processes and growth control (25) and might also be involved in the cold stress signaling (26).

Despite a number of genetic and physiological studies of cold acclimation in higher plants, deeper understanding of how algae are adapting to low temperatures is missing. In recent years, integrative studies of transcript, metabolite and protein data revealed complex biochemical networks and systemic regulation at an organismal level (27–31). Here, we looked at global changes at the metabolome, proteome, and complementary transcript levels under cold acclimation of Chlamydomonas reinhardtii. This species is an ideal model system because it shares most of the metabolic and stress responsive pathways with the higher plants, but at the same time its genome is less complex and most of the gene families have a reduced number of members compared, i.e. to Arabidopsis. Phylogenetically C. reinhardtii is placed between higher plants and animals (32).

In this work we aim to present an integrative study of the cold stress acclimation process in C. reinhardtii during a 5-day time course experiment. We have employed methods of high sensitivity quantitative proteomics (GeLC-LTQ-Orbitrap MS; (33)), metabolomics (GC-MS), targeted transcriptomics (RT-qPCR), physiology, and cell-morphology to describe a complete picture of metabolic, physiological and morphological acclimation, providing insights into cold-stress response in Chlamydomonas. We have found a broad set of responses, from classical stress evidences such us reduced growth and photosynthesis reduction, accumulation of sugars, and membrane composition, to more complex responses involving the identification of specific changes in ribosomes, spliceosomes, and proteasomes, and in sugar signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Strains and Cultures—Chlamydomonas reinhardtii** CC-503 cw92 mt+ (available at the Chlamydomonas Culture Collection, Duke University) cultures were grown in HEPES-acetate-phosphate medium (standard TAP medium in which TRIS was replaced by 5 mM HEPES) supplemented with 8 mM NH₄Cl, at 25 °C or 5 °C with shaking (120 rpm) under continuous light (85 μmol m⁻² s⁻¹; Sylvania GroLux lamps). CC-503 is a cell wall mutant, derived from CC-125 (agg1+, nit1, nit2), which cannot grow on nitrate as a sole N source because of the lack of nitrate reductase (nit1), showing a blocked nitrate-dependent signaling because of the mutation in the regulatory gene nit2. Cultures were prepared 48 h before starting the experiment diluting sixfold a stock culture originated from a single colony. Cells were collected at density 1–6 × 10⁶ cell ml⁻¹ (0–120 h of culture; in exponential phase).

**Physiological Measurements**—At each harvesting time the cell density was measured employing a Thoma counting chamber and the fresh weight was estimated gravimetrically. Photosynthetic rates were measured with an imaging and pulse-amplitude modulation fluorimeter (OSI-FL, Opti-Sciences) after filtering 2 ml of culture onto a 20 mm² surface (Munktell Glass Microfibre Discs).

Total lipids were extracted from frozen pellets with 200 μl of a mixture of chloroform/isopropanol (1:1) and vigorous vortexing for 3 min. Samples were centrifuged (14,000 × g, 5 min, room temperature) and supernatant was transferred to a new tube. The pellet was re-extracted with 500 μl of hexane and vigorous vortexing for 3 min. Samples were centrifuged and the combined supernatants were dried in a speedvac. The amount of lipids was determined gravimetrically. For the starch determination the pellets were disaggregated and washed with 2 ml of 80% ethanol and incubated in boiling water for 3 min. The samples were then centrifuged (10,000 × g, 5 min, room temperature) to precipitate solids and the supernatant was carefully discarded and the pellet was air-dried. The pellet was homogenized into 400 μl of water, and the starch was gelatinized by heating at 100 °C for 10 min. Starch content was quantified using a iodine-based assay using commercial starch as a reference.

**Microscopy—Chlamydomonas cultures were fixed in 3% (v/v) formaldehyde and stained with a lugol solution. Stained cultures were observed under an LSM 780 confocal microscope (Zeiss, Germany) and Z series covering all of the cell height were captured. From these stacks the three images closer to the middle section were selected and used for obtaining a maximum projection using the software Fiji (34). Characteristic projections obtained from cultures at 0, 24, 72, and 120 h are shown in Fig. 1 and supplemental Fig. S1.**

**Quantitative Proteome Analysis (GeLC-LTQ-Orbitrap MS)—Proteins were extracted from frozen pellets (50–70 mg fresh weight). Pellets were disaggregated into 400 μl of extraction buffer (82.5 mM Tris-HCl pH 6.5, 5% SDS (w/v), 10% Glycerol (v/v), 10 mM DTT, 1.2% (v/v) Plant protease inhibitor mixture (Sigma P9599) and incubated 5 min at room temperature. After this time the samples weremixed again by pipeting, incubated 3 min at 90 °C, and then centrifuged at 21,000 × g for 5 min at room temperature. The supernatant was carefully transferred to a new tube and proteins were precipitated by adding four volumes of cold acetone 0.1% β-mercaptoethanol. After 2 h of incubation at −20 °C samples were centrifuged 5 min at 5000 × g at 5 °C. Supernatant was discarded and 2 ml of cold acetone were used to wash the pellet using a pipet for disaggregating the pellet, and then centrifuging 5 min at 5000 × g at 5 °C for pelleting the proteins. This wash step was repeated twice, and then pellets were air-dried. Pellets were re-dissolved in 125 mM Tris-HCl pH 6.8, 4% SDS (w/v), 40% Glycerol, 0.5% (v/v) Protease Inhibitor Mixture, 3% (v/v) β-mercaptoethanol and protein concentrations were estimated employing Bicinchoninic acid assay (35). Proteins were prefracionated by SDS-PAGE. Fifty μg of total protein were loaded into a miniprotein cell and run for 1.5 cm. Gels were fixed and stained with a Methanol:Acetic Acid:Water: Coomassie Brilliant Blue R-250 (40:10:50:0.001). Gels were destained in methanol/water (40-60) and then each lane was divided into two fractions.
Integration of the Metabolomic and Proteomic Stress Phenotype

following a molecular weight criteria (Rubisco Large subunit band was used as reference). Gel pieces were distained, equilibrated, and digested with trypsin as previously described (36). Peptides were then desalted employing Bond-Elute C-18 SPEC plate (Agilent) and concentrated into speedvac. Before the mass spectrometric measurement, protein digest pellets were dissolved in 4% (v/v) acetonitrile, 0.1% (v/v) formic acid. Ten micrograms of digested peptides were injected into an one-dimensional (1D) nano-flow LC-MS/MS system equipped with a pre-column (Eksigent, Germany). Peptides were eluted using a monolithic C18 column Chromolith RP-18r (Merck, Darmstadt, Germany) of 15 cm length and 0.1 mm internal diameter during a 80 min gradient from 5% to 50% (v/v) acetonitrile/0.1% (v/v) formic acid. MS analysis was performed on an Orbitrap LTQ XL mass spectrometer (Thermo, Germany) with a controlled flow rate of 500 nL per minute. Specific tune settings for the MS were as follows: spray voltage was set to 1.8 kV; temperature of the heated transfer capillary was set to 180 °C. Each full MS scan was followed by ten MS/MS scans, in which the ten most abundant peptide molecular ions were dynamically selected, with a dynamic exclusion window set to 90 s.

Raw data were searched with SEQUEST algorithm present in Proteome Discoverer version 1.3 (Thermo, Germany) as previously described (37). In brief, identification confidence was set to a 5% FDR and the variable modifications were set to: acetylation of N terminus, oxidation of methionine and carbamidomethyl cysteine formation, with a mass tolerance of 10 ppm for the parent ion and 0.8 Da for the fragment ion, and a maximum of one missed cleavage. Nonspecific cleavages were not allowed. Four different databases were employed (Chlamydomonas 3.0 protein, 15256 accessions; and Chlamydomonas Augustus 5, 10.1 and 10.2 protein annotations of the Chlamydomonas 4.0 genome release defined by 16888, 16036, and 17114 accessions respectively). Peptides were matched against these databases plus decoys, considering a significant hit when its XCorr score was greater than peptide-charge state +0.5. Protein functions were identified. The proteins were functionally annotated by homology employing BioMart tool available at Phytozome (www.phytozome.org) and the Algal Functional Annotation Tool (http://pathways.mcdb.ucla.edu/algal/index.html; (38)). The final functional annotation was manually supervised because of the discrepancies found between databases for some accessions and sequences were matched to other plant genomes. Reference spectra for all identified proteins are stored in the publicly available proteomics library PROMEX (39, 40).

The identified proteins were quantitated by a label-free approach based on total ion count (41) followed by a NSAF normalization strategy (42):

\[(\text{NSAF})_i = (\text{PSM/L})_i / \sum_{i=1}^{N}(\text{PSM/L})\]

in which the total number of spectra counts for the matching peptides from protein k (PSM) were divided by the protein’s length (L), then divided by the sum of SpC/L for all N proteins. Statistical analyses were conducted according to (43, 44). Proteins were accounted for quantification only if they were present in all of the biological replicates (n = 3) at least of one sampling time, or in five samples corresponding to different times. Missing values were estimated from the dataset employing a sequential K-Nearest Neighbor algorithm. This procedure was only performed if the peptide was consistent (appeared in at least 15 out the 18 replicates, and no more than one value was imputed per sampling time). Protein abundance values were scaled and subjected to Principal Component and Heatmap-Clustering analyses. Classical univariate approaches, Kruskal-Wallis and Pearson’s correlation coefficient, were conducted after a log transformation for normalizing the samples. Differences within treatments were set for a 10% false discovery rate for an alpha = 0.01.

GC-MS: Polar Metabolite and FAME Analyses—The extraction protocol was adapted from (45). Polar metabolites were extracted from frozen pellets (15–25 mg fresh weight) in one ml of cold extraction buffer [methyl:chloroform:1% acetic acid (2.5:1:0.5)]. The samples were homogenized in a Retsch MM400 instrument with the aid of quartz sand. This was followed by centrifugation at 21000 × g for 4 min at 4 °C. The supernatant was transferred to new tubes and 800 ml of water: chloroform (1:1) were added and vortexed. The mixture was centrifuged at 21,000 × g for 2 min at 4 °C, and the upper layer (polar phase) containing water-soluble metabolites was transferred to new tubes and dried completely by speedvac. Samples were derivatized before injection as described in (45).

FAME was extracted from frozen pellets using 1 ml cyclo-hexane. The samples were homogenized as previously described and 400 μl of water were added and mixed with the supernatant. Upper cyclo-hexane phase was recovered after centrifugation, transferred to a new tube and dried by speedvac. Samples were methylsterified with 295 μl tert-Methyl-Butyl-Ether (MTBE) and 5 μl of Trimethyl sulphonium oxide for 30 min at room temperature according to Furuhashi & Weckwerth (46).

GC-MS measurements were carried out following the procedure previously developed (47, 48) on a triple quadrupole (TSQ Quantum GC; Thermo) instrument. In brief one 1 μl of simple was injected per simple, and GC separation was performed on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm) (Agilent Technologies). Oven temperature was increased from 80 °C to 200 °C at 3 °C/min, and then to 250 °C at 10 °C/min, and hold for 3min. Post run condition was holding 300 °C for 4min. In both cases the mass spectrometer was operated in electron-impact (EI) mode at 70 eV in a scan range of m/z 40–600. Metabolites were identified based on their mass spectral characteristics and GC retention times, by comparison with retention times of reference compounds in an in-house reference library. Typical chromatograms are shown in supplemental Figs. S7 and S8.

Peak areas corresponding to each metabolite were normalized by the total peak area in the sample. Normalized areas were scaled and subjected to Principal Component and Heatmap-Clustering analyses. Classical univariate approaches, One-way ANOVA and Pearson’s correlation coefficient, were conducted after a cubic-root transformation of the dataset. Differences within treatments were set for a statistical significance level of 0.05. Because of the low number of comparisons, fifty, false discovery rate was not controlled in the ANOVA analysis.

Nucleic Acid Analysis—The total RNA was extracted using Qiagen RNeasy kit (Qiagen) following the manufacturer’s instruction. DNase treatment was performed on-column using the DNase kit (Qiagen). RNA integrity was assessed by a denaturing agarose electrophoresis and the possible contamination with DNA was checked by PCR. Two micrograms of DNA-free RNA were reverse transcribed using M-MULV reverse transcriptase (Fermentas) and random hexamers following a standard procedure described by the manufacturer.

Quantitative Real Time PCR—Quantitative Real Time PCRs were performed in a Mini-Opticon System (Bio-Rad) following a sample maximization experimental design with three biological and two analytical replicates. Relative quantities and standard errors of relative quantities were determined according to (49). Ubiquitin and Tubulin were selected as endogenous controls after testing the expression stability of receptor of activated protein kinase C (RCK1), actin, tubulin, and ubiquitin in all of the experimental time points with geNorm software (50). Significant differences between sampling times were determined after applying ANOVA to ΔCq values. Relative quantities and expression values are shown in supplemental Table S4. Reaction mixture composition, PCR cycles, primers and Tm temperatures are listed in supplemental Table S5.
C. reinhardtii during a 6 time points course from 0 h to 120 h aiming to monitor both short (5–24 h) and long term (48–120 h) responses. Cells were Lugol-stained.

Bioinformatic Tools—Statistical procedures described above were performed using the software R v2.12 (51) core functions plus the packages SeqKNN, qvalues, agricolae, and gplots. Granger causality of proteins, metabolites, FW, Fv/Fm, starch, and lipids was tested with the statistical toolbox COVAIN (52). Parameter settings for COVAIN were Granger lag time 1 and Granger significance p value 0.01 according to (53). Network analysis was performed with the package mixOmics for R. Network topology was defined after applying a sparse partial least squares (sPLS) regression (54) using proteins as predictors and Fv/Fm, FW, Total Lipids, and Starch as responses. sPLS and PLS methods were compared, also the error of the models were plotted using different number of components. All the variables were kept after the sPLS analysis, for a later filtering based on edges correlations. The network was built using the network function of the same package and the clustered image map was exported to Cytoscape for analysis (55). The network was also visualized and edited in Cytoscape using the network analysis plug-in and using a force-directed layout. Only the edges with correlations higher or lower than ± 0.7 were visualized. Nodes without any edge above the threshold were removed from this analysis.

RESULTS

System Level Changes in Chlamydomonas reinhardtii Following a Decrease in Temperature to 5 °C—We have studied the process of cold adaptation in C. reinhardtii during a 6 time points course from 0 h to 120 h aiming to monitor both short (5–24 h) and long term (48–120 h) responses.

During this period the morphology of the cells changed dramatically (Fig. 1 and supplemental Fig. S1), organelles are progressively disorganized and disrupted, i.e. the nucleolus cannot be distinguished after 24 h, and a complete vesiculizing-granularization can be seen after 120 h of cold exposure, whereas at the same time vacuoles are increased (supplemental Fig. S1A). The chloroplast also changed its shape, and the autofluorescence varied during the time (supplemental Fig. S1A, 1B). The flagella are also shortened with time, after 24 h more than 60% of the cells showed shorter flagella (around half length). This shortening continues with time, and after 72 h, no flagella can be distinguished in more than 80% of the cells, a proportion that is increased to >95% after 120 h.

On the other hand the pyrenoid remains with the same size, but its surrounding starch sheath thickness increases with time. The number and size of starch granules in the chloroplast is also increased with time.

Cold stress affects the growth rate, being significantly slower than compared with 25 °C (Fig. 2A). After 72 h the culture cell density stabilizes and cells increase their volume rather than its number (data not shown). Fv/Fm and total lipid content are also reduced by half but, at the same time, cells starting to accumulate starch (Fig. 2B). A principal components analysis was performed by integrating protein (supplemental Table S1) and metabolite (supplemental Table S2) datasets, starch, lipids, biomass, and Fv/Fm to reveal the most dominant processes and their correlations to specific proteins and metabolites by data-dimensionality reduction (56) (Fig. 2C). The functional interpretation of the principal components (PC1 and PC2) allowed the classification of adaptation processes (Fig. 2C, supplemental Table S3 and summarized in Fig. 3, see also discussion below). PC1 explains the whole variation related to cold acclimation and long-term exposure to cold while PC2 correlates with short-term responses, photosystem proteins, carbon concentrating mechanisms, and starch biosynthesis. This ability to distinguish between groups using our large datasets ensures that we have collected most of the biological variance with low levels of noise and high reproducibility. At the same time the similarity between biological replicates reveals the homogeneity of the cold response between independently grown cultures.

sPLS-correlation network analysis revealed two clusters: (1) a positively correlated cluster of starch and fresh weight (FW) and proteins involved in translation and protein degradation and (2) a cluster of Fv/Fm and total lipids which is negatively correlated to FW/starch (supplemental Fig. S2). Proteins like components of the proteasome complex or carbonic anhydrases connect these different clusters.

Additionally, we performed Granger causality analysis to reveal time-shift relationships between metabolites and proteins during the cold adaptation process using a statistical toolbox COVAIN (52, 53). Granger causality analysis looks for time shift in the correlation patterns (57). This statistical analysis revealed correlations as exemplified in Fig. 3A and 3B not detectable by a simple pairwise correlation analysis. Here, a time-shifted correlation was detected between Fv/Fm and a protein which is annotated as an oxaloacetase (Cre03.g200250.t1.1) hydrolyzing oxaloacetate to acetate and...
Intriguingly, the product of this enzyme oxalate showed the same time-shifted correlation with Fv/Fm. Cre03.g200250.t1.1 is rapidly down-regulated and readjusted to even higher levels. This coincides with the concentrations of oxalate. The role of oxalate accumulation is not clear. A list of Granger correlations is found in supplemental Table S6.

Combined Proteomic, Metabolomic, and Transcriptomic Analysis of Chlamydomonas reinhardtii—For extraction and enhanced protein identification we developed a novel SDS-PAGE prefractionation technique coupled to LC/MS analysis (GeLC/MS), which allowed the identification of 1100 proteins following a database-combinatorial workflow (37). For quantification purposes 708 proteins were filtered out of this data set with efficient peptide counts to ensure accurate quantification purposes. 708 proteins were filtered out of this data set with efficient peptide counts to ensure accurate quantification purposes. Oxalate. Intriguingly, the product of this enzyme oxalate showed the same time-shifted correlation with Fv/Fm. Cre03.g200250.t1.1 is rapidly down-regulated and readjusted to even higher levels. This coincides with the concentrations of oxalate. The role of oxalate accumulation is not clear. A list of Granger correlations is found in supplemental Table S6.

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cation (58). This filtering step, followed by a log-transformation, reduces the mean CV% of technical replicates from 17.43% to 8.71%. Despite the recent advances in Chlamydomonas protein annotation (38, 59, 60) only 552 proteins (76% of the protein IDs; 86.2% of the total protein amount) have been annotated to specific functions (supplemental Table S1), remaining a high number of protein functions to be determined. The approach used in this work, combining different genomes annotation releases (for more details see also (39)), has allowed the annotation of new gene products like Cre04.g211600.t1.2, assigned to be an ortholog to snRK1, which we call CKIN1, Cre13.g570250.t1.2 annotated as CKIN2, or Cre06.g283400.t1.1, a Sucrose-6-phosphate phosphatase, being key steps of its corresponding pathways that were missing from the genome annotation.

A summary of the abundance of each functional group during the time course is shown in supplemental Fig. S3. After univariate statistical analysis the accumulation levels of 451 proteins were considered differential during the time course. The differential concentrations of the annotated proteins as a response to cold stress are shown in supplemental Fig. S4. Biclustering analysis of the proteins shows very accurate distinction of the time and cold stress course differentiating sample groups and functional clusters, demonstrating the dramatic changes induced by cold.

The changes in the primary metabolism were studied by GC-MS, and a total of 54 metabolites were unequivocally identified after the comparison to reference standards (supplemental Table S2). Half of the studied metabolites showed a significant differential accumulation, at least in one of the sampling times. This shows that cold adaptation process require a reprogramming of the major metabolic pathways in Chlamydomonas. Multivariate analysis of the metabolites showed similar characteristics to protein-based classification, indicating key metabolites with high loadings on principal components, like putrescine or glyceric acid (supplemental Fig. S5).

Proteomic and metabolomics analyses were complemented with RT-qPCR with the objective of studying those genes for which its product cannot be detected at protein level and its value is needed for the interpretation of the data. Several genes have been studied covering different metabolic (carbon concentrating mechanisms, sucrose and fatty metabolism) and sugar-signaling pathways (CKIN1, CKIN2, CKIN3) (supplemental Table S4). The integration of metabolites, proteins and transcripts revealed a first multilevel description of C. reinhardtii cold adaptation. The major biological activities during cold adaptation are described below.

**Cold Adaptation Processes of C. reinhardtii in Central Metabolism**—The different pathways related to primary metabolism showed different trends during cold exposure. Changes in enzyme abundance and most abundant metabolites are depicted in Figs. 3 and 5.

The glycolytic pathway is, in general, maintained with most of the related enzymes showing the same abundance level during all the experiment (supplemental Table S1). The enolase (PGH1) abundance increased 1.4-folds after 5 h of cold exposure but quickly decreases to −1.4-folds after 24 h of culture. Phosphoglycerate mutases (PGM1, PGM7) follow the same trend, with an initial increase of 1.5-fold during the first 48 h and then decreasing to control levels (supplemental Table S1). Interestingly one chloroplastic isoform of fructose-1,6-bisphosphate aldolase (FBA1) can only be detected after 72 h of culture.

Glucogenesis seems to be increased with time (supplemental Table S1), because pyruvate phosphate dikinase (PPD2) is increased up to fourfold after 72 h of culture. On the other hand the abundance of phosphohexomutase is constant and fructose-1,6-bisphosphatase (FBP2) decreased with time (not detected after 120 h of culture).

The abundance of most of the enzymes of the Tricarboxylic Acid Cycle (TCA) remain at the same level during all the time points of cold adaptation, with the exception of two isoforms of the enzymes succinate dehydrogenase (SDH2) and pyruvate dehydrogenase (PDH1) showing a constant decrease from 0 to 48 h, and no detection at later time points. Malate and succinate are accumulated (up to +twofold) from 5 to 48 h and then returned to control values. The Glyoxylate cycle, despite sharing most of the enzymes with the TCA, was reduced with time, showing isocitrate lyase (ICL1) and malate synthase (MAS1) isoforms with -fourfold and -sevenfold decrease in abundance after 120 h of culture (supplemental Table S1).

Most of the proteins related to oxidative phosphorylation remained unchanged (supplemental Table S1). Cold stress reduces progressively the abundance of some electron transfer chain proteins, mitochondrial ATPases (ATP1A) and some associated proteins (ASA), NAD/Ubiquinone reductases (-twofold change and loss of some isoforms), Cytochrome C oxidases (COX) (-1.5-fold change), Ubiquinol:Cytochrome C oxidoreductase (QCR9) and inorganic pyrophosphatases (IPY1, IPY3) (-5.2- or −1.5-fold after 120 h), whereas vacular ATP synthases (ATPvA1, ATPvB, ATPvD2) are increased (supplemental Table S1).

Two key enzymes involved in the pentose-phosphate-pathway, 6-phosphogluconolactonase (PGL) and transaldolase 2 (TAL2), were linearly increased reaching a threefold increase after 120 h of culture and a 1.5-fold increase after 48 h of culture, respectively.

The efficiency of photosystems decreased in the first 24 h following cold (Fig. 2B). This can be explained by an initial degradation of part of the light harvesting complex proteins, and b6f cytochromes (supplemental Table S1). Some of these proteins, like the Light-harvesting proteins (LHC) of photosystem I (PSI) (LHCA6, LHCA7, LHCA8) and two chloroplastic ATP synthases (ATPC, ATPD) showed a fast −2.45-fold decrease in abundance. From the detected proteins only three...
did not follow this pattern: LHC-II chlorophyll a/b binding protein (LHCBM3), photosystem II (PSII) subunit 28 (PSB28), and reaction center W (PSBW), for which the abundance increased up to 2.4-fold.

Enzymes belonging to the CO₂ fixation pathway, like phosphoribulokinase (PRK1), ribose-5-phosphate isomerase (RPI1), ribulose phosphate-3-epimerase (RPE1), maintained a constant abundance. The ribulose-1,5-bisphosphate carbox-
Fig. 5. **Central Metabolism.** A, Simplified carbohydrates pathways. Major metabolites and enzymes related to sucrose and glucose metabolism are represented. Starch was accumulated with time due to an increased abundance of starch synthase and ADP-glucose pyrophosphorylase, and decreased amylases. The expression levels of trehalose synthase, sucrose phosphate synthase, and sucrose phosphate phosphatase are up-regulated, defining the accumulation patterns of the studied metabolites. Bars represent relative expression levels at 0, 24, 72, and 120 h. Proteins, transcripts and metabolites were quantified and shown in green, orange and blue colors respectively. B, Increase of specific unsaturated fatty acids in response to cold stress. C, Increase of specific amino acids in response to cold stress.
In a situation of decreased photosynthesis, a very active metabolism, growth and reorganization of the cellular and subcellular structure, the constant supply of carbon to central metabolism should be guaranteed. In this context two phosphoenolpyruvate carboxylases are progressively accumulated during cold stress. Two carbonic anhydrases (CAH1 and CAH3) involved in carbon concentration mechanism were also detected (62). CAH1 had no significant variation during the experiment, whereas CAH3 increased in abundance. This effect was crossvalidated with RT-PCR (supplemental Table S4). The gene expression levels of CAH3 and phosphoenolpyruvate carboxylase (PEPC2) were also increased 4.5- and 2.7-fold, respectively, while CAH1 levels remained stable after 120 h of cold exposure indicating diverse inorganic carbon concentration mechanisms. These effects are also observed for the low CO2 syndrome (63). In parallel, these effects can compensate PS and RuBisCO reduction as observed in our study of C. reinhardtii cold stress adaptation.

**Total Lipid Content is Reduced, but FAME Showed Increased Desaturation**—The lipophilic fractions, containing glycerides, free fatty acids (FA) as well as lipophilic secondary metabolites (e.g. steroids and terpenoids), were decreased progressively up to 72 h of culture and then maintained. Concomitantly, the initial step of fatty acid biosynthesis is significantly up-regulated with an increased abundance of acetyl-CoA-carboxylase (ACC) both at gene and protein levels (supplemental Tables S1 and S4). Although intermediate steps in this pathway, like enoyl-ACP-reductase (ENR), only changed slightly during cold adaptation the enzyme acyl-ACP-thiosterase (ACP) showed a 4.7-fold increase in its transcript levels (supplemental Fig. S6). In addition, an increase of acetyl-CoA thioesterase and acyl-CoA transferase indicates a stimulated FA synthesis. However, this reprogramming is rather involved in qualitative changes of fatty acid profiles (Fig. 5B; supplemental Table S2), with a significant increase of C16:2 and C18:2 (5?,9) in detriment of C18:0. To further investigate these observations, gene expression levels of four desaturases were studied (supplemental Fig. S6; supplemental Table S4). Stearoyl-CoA desaturase (Δ9; SCD), the desaturase that insert the first double bond (C16:1(9); C18:1(9)), increased threefold after 24 h of cold exposure and Δ5 desaturase (DD5) increased 2.5-fold after 120 h of cold exposure. Desaturases Δ12 (CDD12) and Δ9 (DD9) did not significantly change during the experiment. In consequence C. reinhardtii cells reprogrammed the FA biosynthesis pathway very specifically as a response to cold exposure. Given that the conservation of FA biosynthesis, and desaturation is crucial for preserving cell energy states (64) and membrane fluidity (5), which is related to cell function and the perception of environmental signals (65, 66) this mechanism indicates how FA biosynthesis and desaturation systems might have evolved for adaptation to cold temperature (4).

**Starch, Mono and Di-saccharides are Accumulated With Time**—Cold stress has been demonstrated to lead to the accumulation of starch but also lead to changes in other sugars and sugar alcohols (Fig. 5A and supplemental Table S2) which represent not only energy and storage products, but also being carbon precursors, transport compounds, and signaling molecules.

The accumulation of starch is clearly explained by increased ADP-glucose pyrophosphorylase (APL1) and starch synthase levels. At the same time a reduction of amylases is observed (Fig. 5A). Intriguingly, not the soluble starch synthase (STA1) but granule-bound starch synthase (SS1)—not detected in controls—increased constantly from 5 h of culture (4-folds increase at 120 h compared with 24 h). APL1 followed the same trend with significant up-regulation of the gene and accumulation of the protein during the experiment. a-amylase (AMA2) was only detected in the first 24 h. The detection of these enzymes after 24 h of culture indicates that starch accumulation is a quick adaptive mechanism, most probably as a metabolic switch for storage compound synthesis.

The disaccharides sucrose and trehalose showed a decrease in concentration at five hours of culture, but then both showed a trend of accumulation of 1.16- and 1.48-fold respectively. All of the genes involved in sucrose and trehalose pathway are significantly up-regulated during the experiment following a correlated expression pattern (Fig. 5, supplemental Fig. S6). The coordination of biosynthetic gene expression and metabolite accumulation of sucrose and trehalose through an increase in concentration at five hours of culture indicates that starch accumulation is a quick adaptive mechanism, most probably as a metabolic switch for storage compound synthesis.

The accumulation of sugar alcohols sorbitol and myo-inositol were increased and behaved differentially. Sorbitol accumulated (+1.5-folds) at 24 h of cold exposure, returning then to control levels. Myo-inositol had a maximum increase of twofold at 120 h. The accumulation of these compounds is suggested to have a thermo and osmoprotective function (67), however, the protective effect of these metabolites may be limited in Chlamydomonas, because the increase in abundance stress is only small (twofold maximum).

**Protein Biosynthesis is Increased and Splicesomes and Ribosomes are Remodeled**—The basis for a stress adaptation is a specific regulation of the response, starting with RNA expression, RNA processing, protein translation and ending with post-translational modifications. These changes may lead not only to the production of the needed proteins, but also to specific isoforms that are more favorable to cold environments. Prohibitin (PHB), which regulates transcription by an interaction with different transcription factors and maintain mitochondrial function, is accumulated with time (+2.5-fold at
120 h). CAPER, which regulates both steroid receptor-mediated transcription and alternative splicing (68), was repressed after 48 h of cold exposure. This down-regulation coincides with lower levels of steroids in the lipid fractions (see above), however, the specific functions of these proteins are not known in Chlamydomonas.

At spliceosome level initial cold stress induced the expression of the Splicing factor 3 subunit 1 (SF3A1) and two members of the U1 snRNP splicing factor family (PRP39), which can be detected after 5 h of culture. Alternate splicing is probably used by the Chlamydomonas as a quick adaptive mechanism for producing protein isoforms adapted to cold. The spliceosome remodeling has been described as a mechanism to stress adaptation in mammals (69). Stress-dependent and differential pre-mRNA processing has been proposed as a new mechanism of stress response and regulation (70).

Temperature-dependent splicing has been shown for Arabidopsis SR1 splicing factor (71), and differential pre-mRNA processing has been related to cold stress adaption in Arabidopsis (74). Stress-dependent changes in the ribosome. The adaptation of the ribosome to the stress conditions would be the next step for a more efficient and targeted protein biosynthesis. Families of the large (L10a, L13, L22, L36) and small (S3, S6, S7, S8, S9, S10, S17, S19, S21) subunits of the ribosome are accumulated, both at cytoplasmic and mitochondrial/plastid levels. In this context changes of subunit S6 and its phosphorylation has been related to low temperatures.

The cold-induced alternate-splicing is also followed by changes in the ribosome. The adaptation of the ribosome to the stress conditions would be the next step for a more efficient and targeted protein biosynthesis. Families of the large (L10a, L13, L22, L36) and small (S3, S6, S7, S8, S9, S10, S17, S19, S21) subunits of the ribosome are accumulated, both at cytoplasmic and mitochondrial/plastid levels. In this context changes of subunit S6 and its phosphorylation has been related to low temperatures.

The microalgae C. reinhardtii undergoes drastic changes in metabolism and development when cold stress is applied (these changes are summarized in Fig. 4). Despite the suitability of this species for studying temperature stress (stress can be applied homogenously and rapidly to all cells, vegetative cells are undifferentiated, the genome is sequenced (82), gene families are smaller compared with higher plants), to our knowledge, there are no studies about cold stress in C. reinhardtii. Former studies in plant biology have described mechanisms implied in the cold response such as metabolomic changes (30, 53, 83–85), integration of metabolomic and proteomic phenotypes (30), anatomical changes, sugar accumulation (15, 26), late embryogenesis abundant (LEA), and heat shock (HSP) proteins mediating stabilization of membranes and proteins (65, 86), and the activation of C-repeat-binding factor and dehydration responsive element-binding factor 1 (CBF/DREB) responsive pathways (2, 9). Most of these studies have been focused on individual mechanisms with comparison of control versus stressed plants. Here, we aim for systemic analysis, integrating metabolite, protein and transcript data to characterize cold induced changes during a...
Cold Acclimation—

*C. reinhardtii* respond to cold stress by a progressive adjustment of its metabolism with sustained, transient, early- and late-responsive metabolic alterations.

**Cell Organization and Membranes are Remodeled after Cold Acclimation**—The increased vesicularization of the *C. reinhardtii* cells observed during cold stress exposure can be an indicator of progressive membrane damage, since cell vacuolarization has been reported as reporter under other stress situations (87). Furthermore, the rigidification of the membranes has been proposed as one of the first steps in cold stress signaling in *Arabidopsis thaliana* (5). This phenomena is also observed in *C. reinhardtii* (see Fig. 5B). Other general responses to cold-induced membrane damage are an increased accumulation of cryoprotectants (12, 13, 15); LEA, cold- and heat-shock proteins (HSP), which participate in membrane protection and refolding of denatured proteins (88, 89); and the accumulation of antioxidants mostly at chloroplast level (90, 91).

Interestingly members of the glutaredoxin, thioredoxin and redoxin families were inhibited by cold, while ferredoxins were accumulated. The change in the rate of the different antioxidant enzymes is an example of cold induced proteome remodelling. Thioredoxins are labile to low temperatures, with a high unfolding constant (6), so its quick denaturation may lead to degradation and recycling, while ferredoxins, with a much more lower unfolding constant and resistant to temperature changes, became over-accumulated. These biophysical properties of ferredoxins may explain its known function of controlling oxidative damage and maintaining the integrity of thylakoid membranes and photosynthesis during cold stress (92, 93). The ferredoxin and thioredoxin system is reported to have also important roles in light perception and redox signaling pathways within the chloroplast (94).

One particular observation related to the membrane maintenance was the accumulation of vesicle inducing protein in plastids 1 (VIPP1) and Thylakoid formation proteins (THF) that are part of a mechanism related to thylakoid membrane formation, remodeling and vesicular trafficking (95). These proteins are strongly accumulated after 48 h of cold exposure which is coincident with the vesicularization of the chloroplast (Fig. 1, supplemental Fig. S1). None of these systems have been reported so far to mediate the cold stress response in Chlamydomonas. Therefore they are excellent candidates for further functional studies of *C. reinhardtii* and general algal cold stress adaptation. Furthermore the LHC interacting protein stress-related a/b binding protein 1 (LHCSR1), previously related to sulfur withdrawal (96), was also accumulated. These membrane remodelling and protective mechanisms, together with the partial recovery of LHC and ATP synthesis proteins, are correlated in time to the slight recovery of the photosynthetic rate observed after 72 h of cold exposure, being part of the adaptive mechanism to cold.

**Cold-induced Metabolic Adaption and Signaling in Chlamydomonas reinhardtii**—The response to unfavorable cold condition in *C. reinhardtii* is dynamic and multifaceted. The metabolic adjustment of *C. reinhardtii* did not follow the common response patterns previously described in plants. Organic acids, TCA intermediates and its related enzymes are decreased under cold stress in an opposite way to *Arabidopsis* which tends to activate these pathways (87). Furthermore, on the contrary to other plants in which starch is quickly depleted under stress situations most probably for the conversion into other sugars (19), in *C. reinhardtii* starch is constantly accumulated. This can be explained by a significant increase in SSI and APL1 enzymes and transcripts whereas amylase activity is reduced. It has been demonstrated (98) that trehalose-6-phosphate also regulates starch accumulation by a redox activation of APL1. The accumulation of starch is a pronounced response of *C. reinhardtii* for adaptation to cold environments and obviously not opposed to the accumulation of other sugars. Furthermore the correlation found in gene expression and protein accumulation levels of the analyzed enzymes suggest a possible coregulation of this pathway (99).

The storage and transport of nitrogen as well as the urea cycle is also affected by cold stress. The first response to cold is a quick accumulation of nitrogen in the form of Asp, Arg, Phe, and Glu with a remarkable transient increase of abundance up to +6-folds after 24h in the case of Glu (Fig. 5C). This initial accumulation of amino acids is a key process for recycling cellular components, and may be a hint of a larger autophagy program (100). Starvation was reported to modulate the activation of many genes related to amino acid catabolism and accumulation in Arabidopsis (101). After 48 h of cold exposure the accumulation of these amino acids quickly decreased to control levels. The abundance of urea, that can be used for monitoring the nitrogen balance (102), is also sharply decreased after 48 h of culture. The reduction in amino acids and urea suggests a nitrogen deficiency for *C. reinhardtii* after 3 days of cold exposure, coincident with an increase of the biosynthetic proteins present in the cells, that suggests that nitrogen demand cannot be supplied by cell-recycling processes and also indicating that the nitrogen uptake from the media is dramatically reduced by the cold stress as it is described for *Synechococcus* (103). The fast accumulation of Arg and Glu might explain some immediate response to cold as observed in other plants (104), however, the abundance of all the analyzed amino acids followed specific trends. Remarkable is the initial reduction of the Pro and Gly, common stress markers in higher plants. Their levels only recovered after a 72 h of cold exposure.

**Cold Acclimation in Chlamydomonas reinhardtii Share Elements With Other Stress Mechanisms**—The obtained results
Common stress symptoms such as a reduced activity of light harvesting complexes, and its related decrease in Fv/Fm, TCA cycle activity, accumulation of sugar alcohols and other cryoprotectants and an increased photorespiration are also manifested under cold temperature as in many other green organisms (2, 8). But on the other hand Pro and the polyamine putrescine, common stress markers in other plants (28, 30), were reduced during cold stress whereas protein biosynthesis and degradation and RNA pathways were differentially regulated pointing to some divergences to common stress markers in higher plants.

The first look at the C. reinhardtii cells demonstrates a significant increase of the starch sheath surrounding the pyrenoid while the size of the pyrenoid remained constant. The elements of the Carbon Concentration Mechanism (62) PEPC and CAH are also accumulated during cold acclimation (105). This is a typical response to low CO2 levels, in which the cells aim to concentrate all of the available Ci into the pyrenoid for increasing the efficiency in the carbon fixation. In this particular situation we hypothesize that the increase in these enzymes compensates the loss of activity related to the low temperatures. In contrast, aspartate and glutamate, recently reported to be decreased during under low CO2 (106), are two- and sixfold increased after 24 h following an opposite trend. Furthermore the low CO2 induced proteins LCIB, shown to function downstream of CAH3 in the CCM in the vicinity of the pyrenoid (107), and LCI5, a stromal protein with unknown function (108), reported to be constantly expressed during non CO2 enriched cultures situations are reduced during cold exposure. Therefore the obtained results indicate significant differences between the physiological responses to cold and low CO2, but also suggesting an impairment between the carbon uptake, fixation, and sensing mechanisms under cold stress.

The accumulation of osmolytes like sugars and sugar alcohols are also a typical response to drought and saline stresses avoiding loss of intracellular water and protecting proteins and membranes (8, 19). In higher plants the tolerance to these stresses is also enhanced by DExD/H box RNA helicase protein family (109, 110) with members of this family involved in cold tolerance (111). We have found the accumulation (+2.3-folds) of the product Cre10.g427700.t1.1, a non-annotated gene with DExD/H box RNA helicase domains. The function of RNA helicases are still poorly understood in plants (109), but under cold temperature these helicases have been found to be possibly involved in mRNA export (110) and in increasing the translation efficiency altering the gene expression (112).

The autophagy pathway described to be present in most of the stress responses (76), seems to play an essential role in cold adaption. The autophagy can be triggered by an increased concentration of reactive oxygen species, protein unfolding and the energy deficit (21, 76, 77) symptoms that Chlamydomonas cells suffer after cold exposition. Proof of an increased autophagy are the accumulation of amino acids, the initial decrease on biosynthesis related proteins and the reduced cell division, characteristic symptoms of the inhibition of TOR (75, 77). Sugars and starch are constantly accumulated during the cold exposure, and upturned catabolic activities can be detected after 72 h. This may suggest that TOR is still controlling starch production in Chlamydomonas, giving importance to a recently proposed TOR independent signaling pathway, controlled by AMP dependent kinases (in plants SnRK1) proteins in mammals (113). This pathway, which combines a functional TOR and autophagy, allows the simultaneous accumulation of starch and trehalose and protein degradation pathways showing the complexity of energy-related metabolic networks. Further research is needed to validate this hypothesis.

Sugar Signaling Mediates Cold Acclimation in Chlamydomonas reinhardtii—Stress adaption is a complex process of many different signaling inputs originated in different organelles and pathways that lead to changes in the proteome, modulating the metabolome and in consequence, the physiological response. The connection between the stress sensing and the energy pathways can lead to extensive changes, highly precise, for maintaining homeostasis, promoting cell survival, and establishing the basis for long term adaption (21). This connection is based on the activity of three key regulators: HXKs, snRK1 family (AKIN10 and AKIN11 genes in Arabidopsis thaliana) and TOR. These kinases regulate the activation of different enzymes and transcription factors, not only related to metabolic regulation but also to protein biosynthesis and cell organization, and have a complex regulatory system (22).

The CKIN1 gene—the AKIN10 homolog in Chlamydomonas reinhardtii—is immediately and strongly down-regulated after cold exposure with expression levels recovered after 72 h whereas the expression levels of CKIN2 remained constant. The expression patterns of CKIN1, CKIN2, and CKIN3 in Chlamydomonas are different in cold stress compared with nitrogen depletion or drought stress (Valledor et al., unpublished results) suggesting specific regulation of this stress. The increase of the protein biosynthesis and glycolysis pathways during the first 72 h of cold acclimation, and its later repression when CKIN1 levels are recovered give clues of its function in our experimental system. It has been reported that all of these pathways are repressed in Arabidopsis by AKIN10 (23) while activated in mutants (114). The silencing of AKIN10 was also related to an activation of sucrose-6P-synthase (SPS) and THF1 (which function was described above) and at the same time to a repression of transcription factors and some protein degradation regulators (114). It has been described that different growth conditions and stresses may lead to a differential sensibility of these proteins to their di-
Integration of the Metabolomic and Proteomic Stress Phenotype

ferent regulators (20) adding one more layer of complexity to this regulatory network.

Consequently, the observed changes in gene expression induced by a repression of CKIN1 might reflect a very con-
served convergence of multiple types of stress by energy
deficiency. Chlamydomonas follows the general scheme
found in higher plants showing also some specificities, that
in the particular case of cold stress seems to be regulated by an
increased sugar accumulation. This cold-induced sugar ac-
cumulation, together with other inputs, may tune this general
response mechanism not only to promote common stress
responses, but also specific to cold. In this work we present
some hints and clues of sugar signaling pathways as a basis
of further experiments that will allow a deeper understanding
of stress response mechanisms.

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