SI Appendix

Linking toxicity and adaptive response pathways across the transcriptome, proteome and phenotype of *Chlamydomonas reinhardtii* exposed to silver

Smitha Pillai[1,2], Renata Behra[1,2], Holger Nestler[1], Marc Suter[1,2], Laura Sigg[1,2], Kristin Schirmer[1,2,3]

[1] Department of Environmental Toxicology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Duebendorf, Switzerland; [2]Department of Environmental Systems Science, Eidgenössiche Technische Hochschule, 8092 Zürich, Switzerland; [3]School of Architecture, Civil and Environmental Engineering, École Polytechnique Fédérale, 1015 Lausanne, Switzerland

SUMMARY OF SUPPORTING INFORMATION APPENDIX

Material and methods

Medium and Growth conditions

To avoid metal contamination, all polycarbonate and teflon flasks were soaked for 24 h in 0.03 M HNO_3 and rinsed with deionized water. Cellulose nitrate filters (pore size 0.45 µm; Sartorius AG, Germany) for bioaccumulation experiments were boiled in 0.03M HNO_3, rinsed in water and dried at 50°C for 24 h. *Chlamydomonas reinhardtii* (CC-125, wild type mt+137c) were grown at least for three successive subcultures in modified Talaquil medium (with no chloride salts) before each experiment. The medium was prepared with no chloride salts to avoid forming silver chloride complexes and that most of the silver occurs as free silver ions. The growth conditions were 25°C with constant agitation and illumination of 100 µmol photon m^{-2} s^{-1}. The cell density of the culture and cell size was determined using the Z2 Coulter particle count and size analyzer (Beckman Coulter, USA).

Photosynthetic Yield

The photosynthetic activity of *C. reinhardtii* was measured using the Phytoplankton Analyzer Phyto-PAM (Walz, Germany). It is based on the principle of selective amplification of a fluorescence signal, utilizing intense short pulses of light. The photosynthetic yield was calculated using the relationship, Yield (PSII) = $(F_m\text{-}F)/F_m$, where F_m is the maximum Fluorescence and F is normal Fluorescence

Cell Viability and Growth

Cellular ATP content is an indicator of metabolically active cells. BacTiter-Glo Microbial cell viability assay kit (Promega) was used to determine the amount of ATP in algae exposed to different concentrations of silver for varying durations. To a 100 µl aliquot of the culture, 50 µl of BacTiter-Glo reagent, which contains an extracting reagent and thermo-stable luciferase, was added. The luminescence signal, which correlates to the amount of ATP, was measured after silver exposure. The growth of *C. reinhardtii* was estimated using the Z2 Coulter particle counter The growth rate per hour (µ h^{-1}) was calculated as follows; $\mu = (\ln N_t - \ln N_0)/t_n$, where N_t is the final cell density (cells ml^{-1}), N_0 is the initial cell density and t_n is time in h after the initiation of the test.

Lipid peroxidation quantified by thiobarburic acid reactive substances (TBARS) assay

TBARS, which is a byproduct of lipid peroxidation caused by ROS, was detected using thiobarbituric acid as a reagent (Sigma Aldrich, Switzerland). The assay measures malondialdehyde(MDA), generated from lipid hydroperoxides. Algae were exposed to silver and to thiobarbituric acid at a final concentration of 2.5 mM along with 100 mM trichloroacetic acid in ethanol. The reaction mix with algae was heated to 95°C for 25min and then cooled on ice. The absorbance of the supernatant was measured at 532 nM with a plate reader (Tecan Instruments, Switzerland).

Accumulation of lipid bodies

Lipid bodies of *C. reinhardtii* were stained by exposing the algal cells to Nile red at a final concentration of 1µg ml^{-1}. The cells were imaged using a Leica TCS-SP2 confocal microscope and a 63x oil objective.

Bioaccumulation

The filters with algae were dried, transferred into teflon flasks with 4 ml of 65% HNO_3 and 1ml of 30% H_2O_2, and digested by microwave for 15 min in a high-performance instrument (MLS, Germany). The solution was then transferred into a graduated flask and the volume made up to 25ml with deionized water. A 1:10 diluted sample was used for high resolution-ICP MS analysis (Thermo Finnigan, Germany). To control possible contaminations, several blanks (medium-, filter-, acid-, ligand-, and deionized nanopure water-blanks) were always measured in parallel.

Microarray design and experiment

Total RNA was reverse transcribed to cDNA and cDNA used for the synthesis of cRNA labeled with Cy3 using the one-color Quick Amp Labelling Kit. Hybridization was performed according to the manufacturer's protocol. Each exposure condition and controls were done as three biological triplicates. The Arrays were scanned using an Agilent Technologies Scanner G2505B at 5mm resolution and feature extraction was performed using Feature Extraction software. Additional data analyses were performed with the R/Bioconductor software using background-corrected and quartile-normalized median signals. Significant differences of average signal intensities between conditions were determined by one-way ANOVA using independent variables at different treatments. The data with a cut off for FDR of <0.1 and p<0.001 were considered for further analysis. The annotation of the proteins was done using the database *Go*FORSYS – *ChlamyCyc* (http://chlamyto.mpimp-golm.mpg.de/chlamycyc/index.jsp). The proteins were mapped using the freely available tool MapMan (1).

Proteome Analysis

Protein extraction and tryptic digestion

The algal pellets were lysed in buffer containing 25 mM Tris/HCl pH 8; 25 mM KCl; 25 mM $MgCl_2$ & 0.1% CHAPS and sonicated on ice four times for 30s each. The cell debris was removed by centrifugation and the protein was precipitated using methanol/chloroform. The dried protein pellet was resolubilized in buffer containing 9 M urea, 2 M thiourea, 50 mM Tris-HCl at pH 8.5 and concentration measured using the Bradford assay. 100 µg of total protein was reduced and alkylated using tris 2-carboxyethyl phosphine and iodoacetic acid, respectively and trypsin digested overnight before being quenched by formic acid.

2D Liquid chromatography separation and mass spectrometry of peptides

Tryptic digested proteins (10 µg) were pressure loaded onto a three-phase MudPIT column packed successively with C18 3 µm (analytical column), SCX on 5-µm beads, and C18 5-µm (pre-column) resins. The column with the peptides was desalted and placed in a nanoelectrospray ionization source on the LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) for elution. The 11-step MudPIT protocol (2) was used for elution of the peptides. The instrument settings are described in the supplementary information (SI Appendix). For each exposure condition, three biological replicates were analyzed. A label-free spectral count approach (3) was used for quantitation, followed by statistical evaluation by G-test (4). The instrument was run in positive ion mode with a 1.2 kV spray voltage, with the tube lens set to 110V and ion transfer capillary temperature to 200°C. One full scan FT mass spectrum (400–1600 *m*/*z*, resolution of 60 000) was followed by seven data-dependent MS/MS scans acquired in the linear ion trap with normalized collision energy (setting of 35%) and continuously repeated throughout each MudPIT step. Dynamic exclusion was activated for 120 s and data-dependent acquisition was triggered by the most intense peaks determined to be carrying 2 or 3 positive charges.

Analysis of LC-MS/MS data

The MS raw files were peak listed with extract_msn.exe (X-Calibur Thermo Fischer Scientific, Switzerland) followed by merging of the MS/MS peak lists to .mgf format by perl script merge.pl (Matrix Science, UK). The analysis of the data was done as described previously (2). The Open Mass Spectrometry Search Algorithm (OMSSA, version 2.1.7) was used to search for peptide hits in curated database. It includes the Chlamydomonas JGI 4 'best protein' containing approximately 15000 nuclear and, 76 chloroplast and mitochondria encoded proteins from Chlamycyc (http://chlamycyc.mpimp-golm.mpg.de/files/sequences/protein/). The criteria for selection of the peptide sequences are described follows. Sequences of less than 10 amino acids were removed from the databases. The FDR was estimated with a reversed version of the database created with decoy.pl (Matrix Science). The modifications set for the database search were carbamidomethyl-cysteine (fixed) and N-terminal acetylation and formation of pyroglutamic acid (variable). After recalibration of systematic mass errors, the precursor-ion mass accuracy was set to 0.005 Da. The peptide hits with the lowest E-values and FDR <0.5% were considered for further statistical analysis. Results from the biological triplicates were merged and spectral counts were calculated from the number of discrete peptides for each protein. The statistical significance was calculated by G-test as described previously (2, 4). The annotation and enrichment analyses of significantly regulated proteins were done using the same databases as for the transcriptome analysis.

Table S1: Transcriptome probe counts by significance and fold-change (fc) analysed by Multi group ANOVA analysis. We analysed genes regulated with a fold change above 2 (**fc >= 2)** with **p < 1e-04**

	fc >= 1	**fc >= 1.5**	**fc >= 2**	**fc >= 3**	**fc >= 4**	**fc >= 8**	**fc >= 10**
p < 0.1	13252	12660	10377	7197	5334	2134	1499
p < 0.01	11731	11432	9725	6943	5194	2096	1477
p < 0.001	10383	10247	9031	6655	5053	2060	1448
p < 1e-04	9032	8983	8211	6308	4884	2024	1420
p < 1e-05	7804	7777	7290	5819	4586	1956	1376

Figure S1: The figure shows the heat map of the transcriptome based on two-group analysis at different silver exposure conditions. Each bar represents a transcript, with green being down and red upregulated. The white bars represent transcripts abundance close to that of the control.

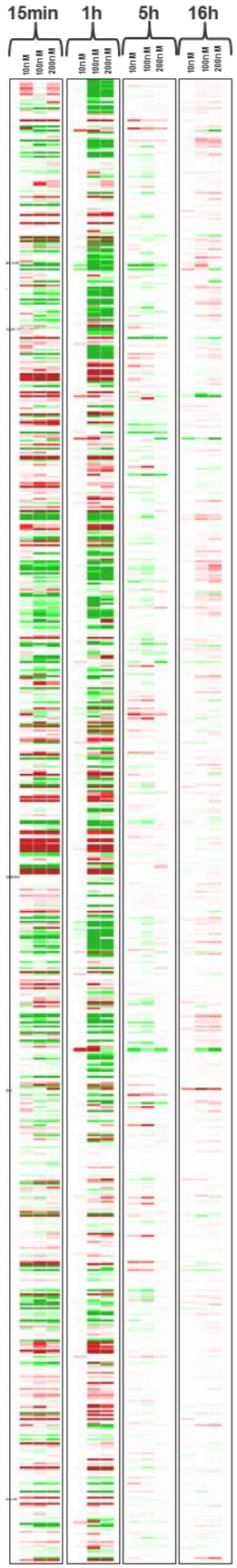


Figure S2: Number of commonly regulated transcripts (log 2 fold change of >1 or <-1) at time points 15min and 1h

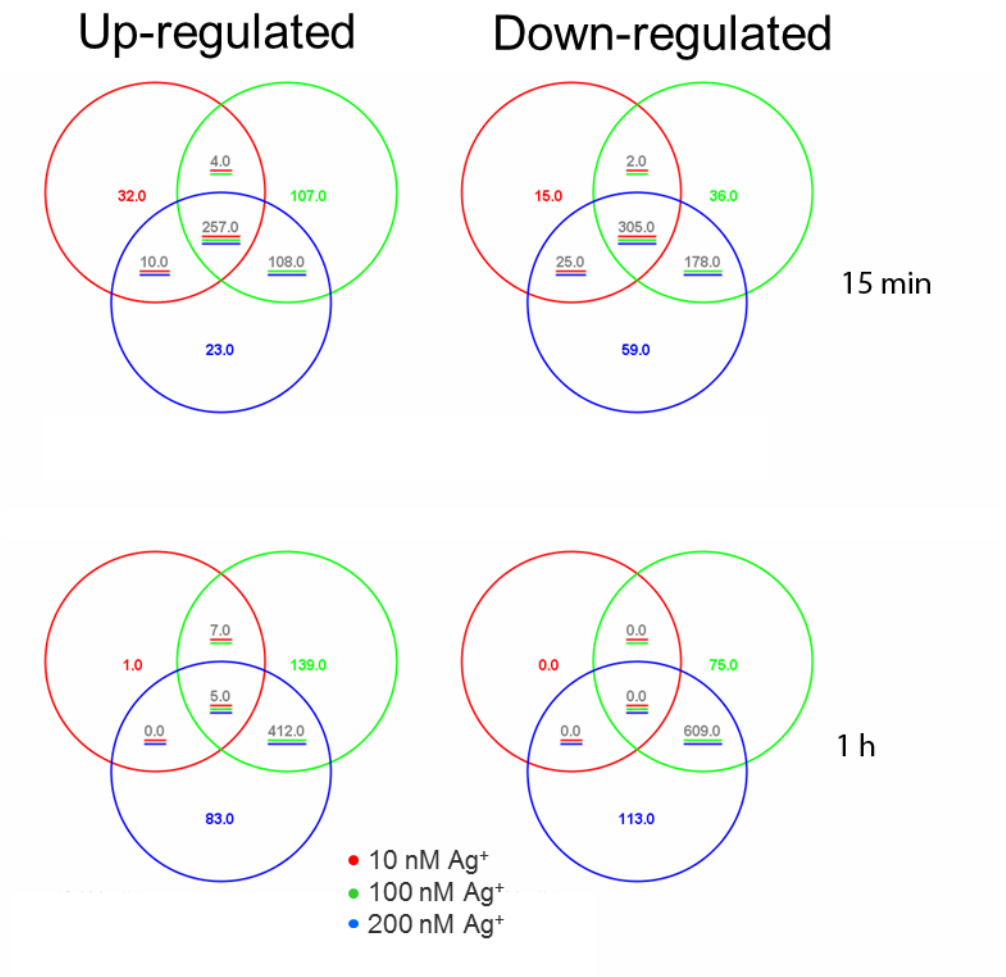


Figure S3: Heat map of the proteome based on two-group analysis at different silver exposure concentrations for 1h and 5h. Each bar represents a protein, with green being down-regulated and red upregulated. The white bars represent protein abundance close to that of the control.

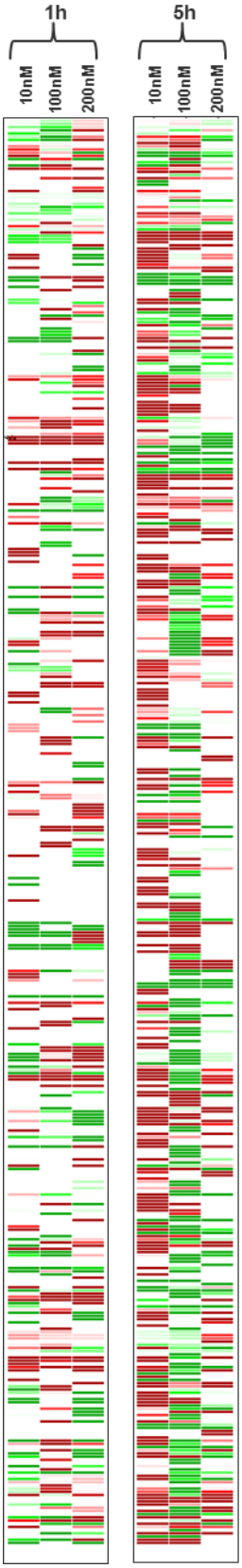


Figure S4: MapMan ontology term enrichment of commonly regulated proteins at the transcriptome and proteome level. The bars represent the enrichment score of the proteins (y-axis) in different biological functions (x axis). Results demonstrate that, though several pathways were commonly regulated at the level of the transcriptome and the proteome, the extent of enrichment differs

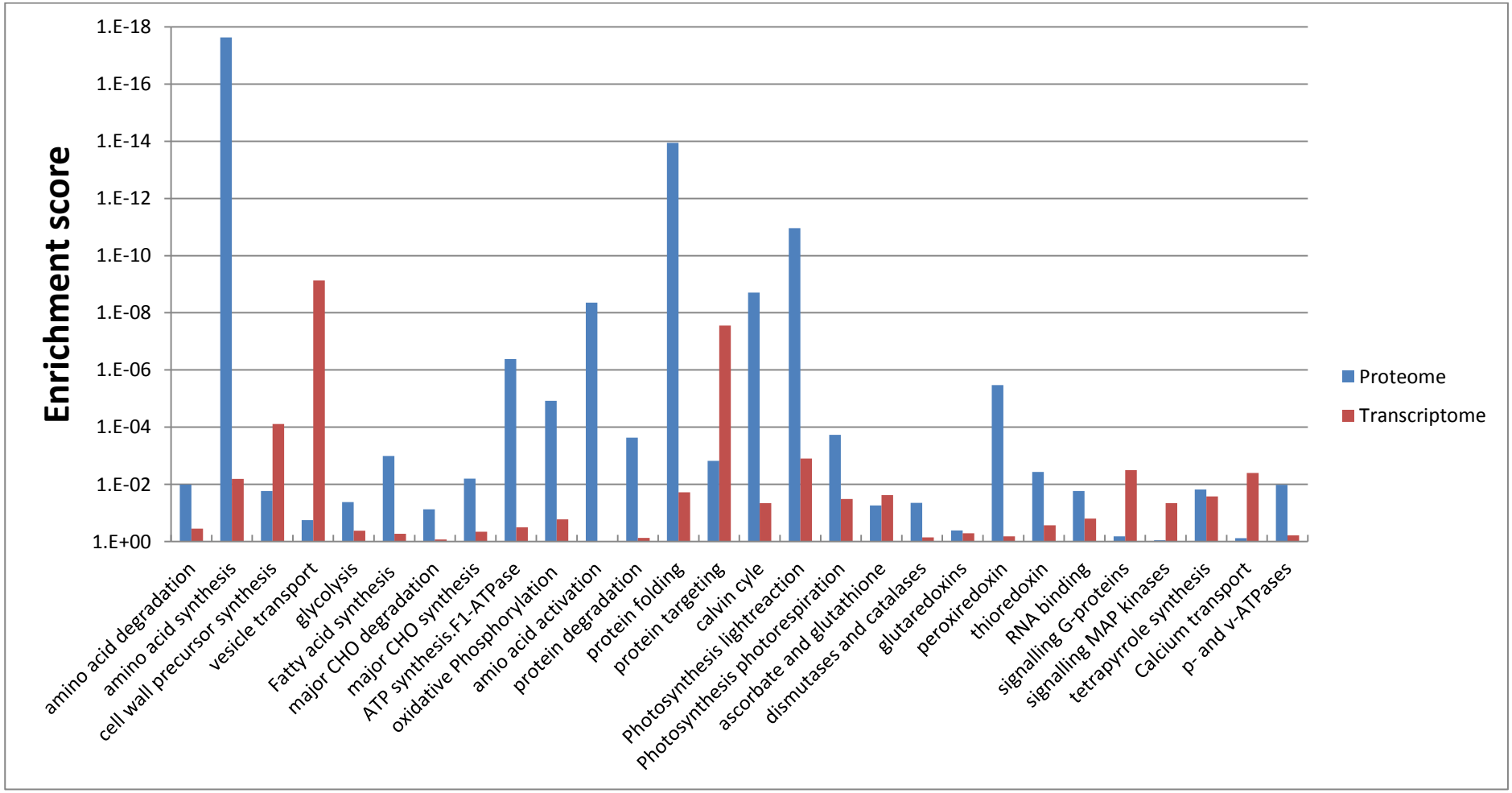


Figure S5: MapMan ontology term enrichment of proteins regulated exclusively at the proteome level, with the enrichment score on the y axis and MapMan functional categories on the x axis.

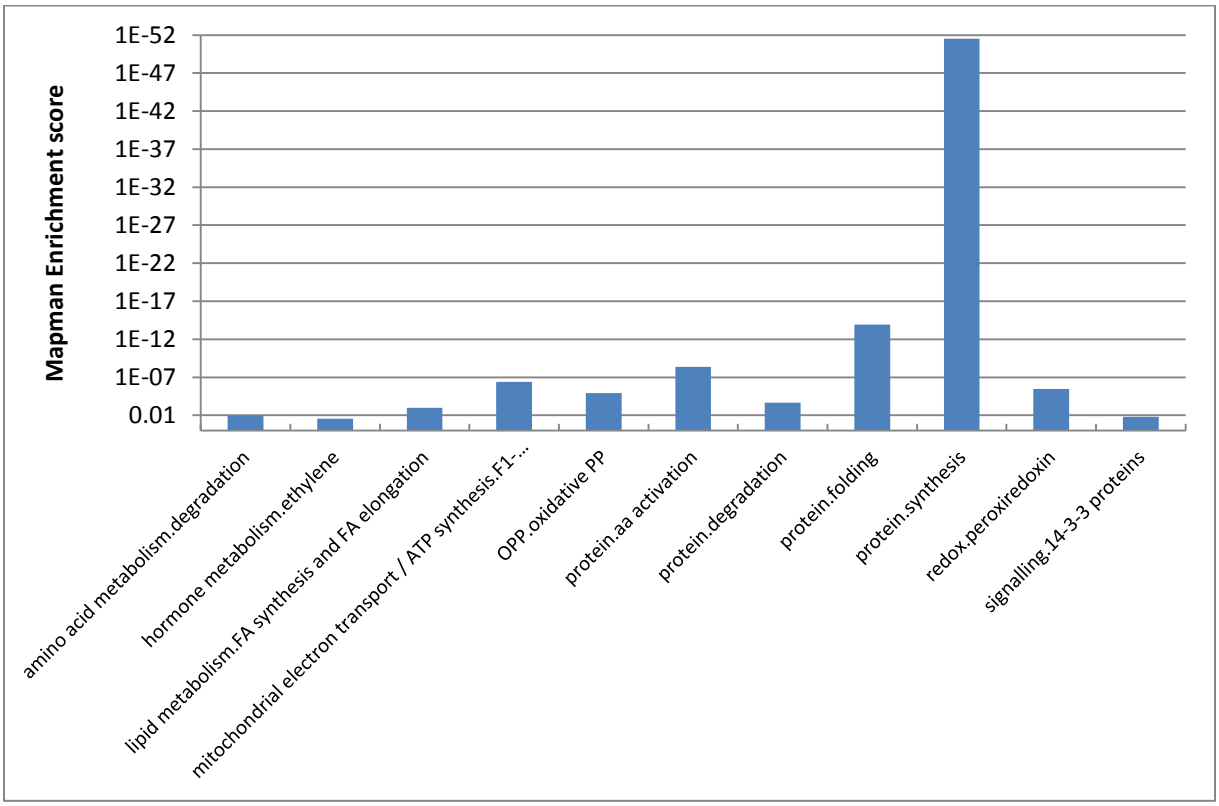


Figure S6: MapMan ontology term enrichment of significantly regulated transcripts only at the transcriptome level, with the enrichment score on the y axis and MapMan functional categories in the x axis

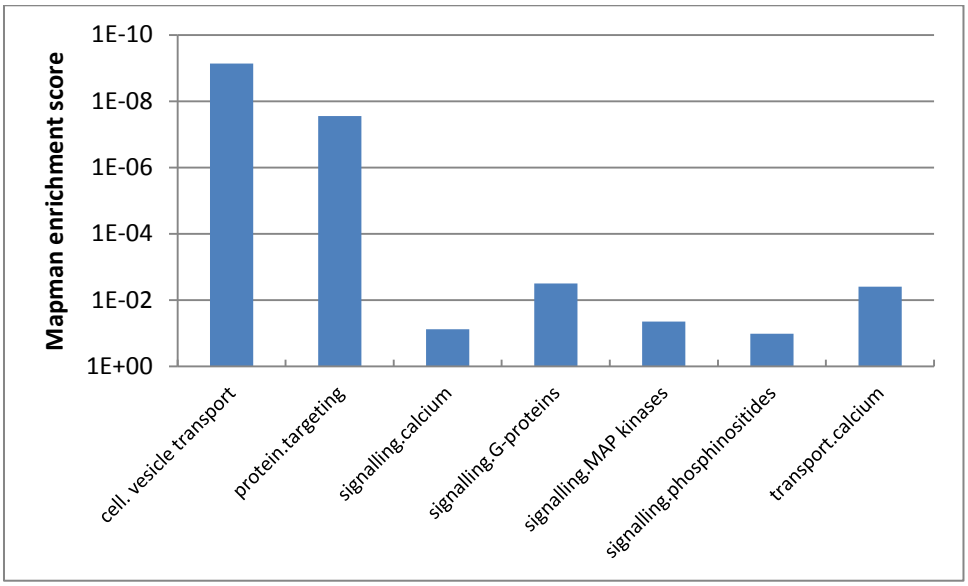


Figure S7: MapMan graphical representation of the regulation of the photosynthetic machinery exposed to 200nM Ag^{+} for 1h at the transcriptome level. Each green or red box represents a transcript with green being down-regulated and red being up-regulated.

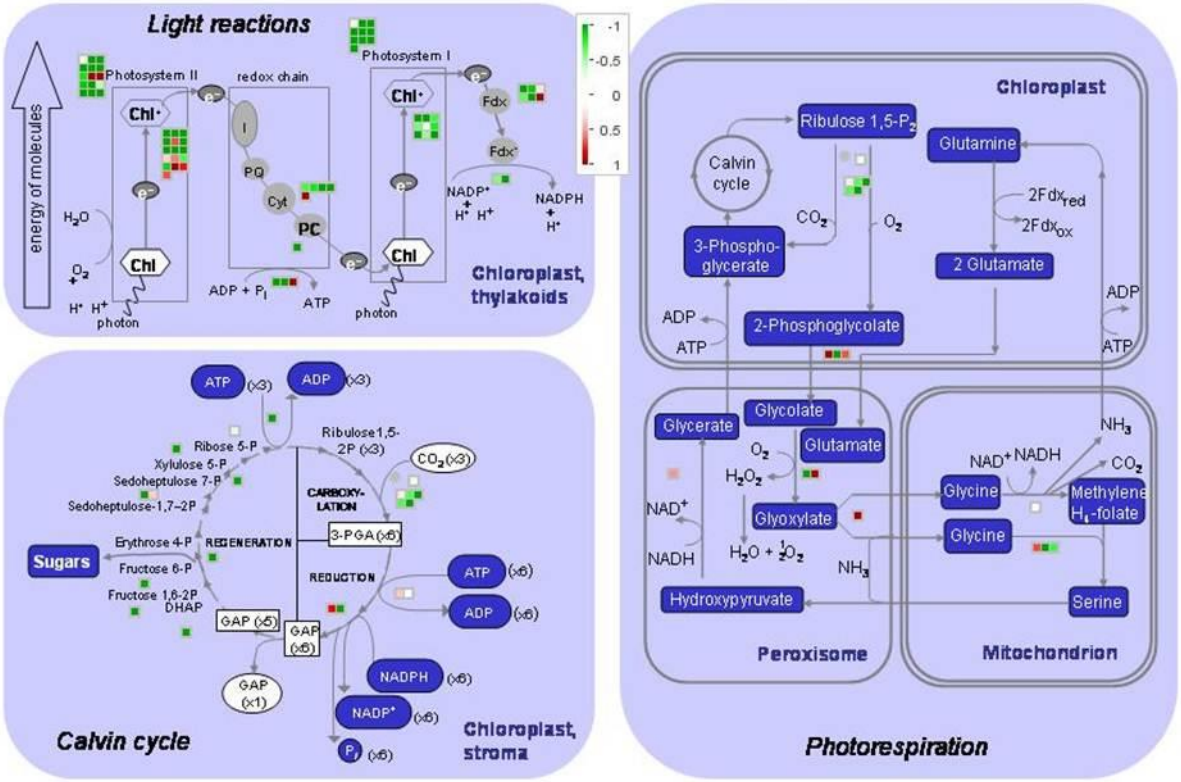


Figure S8: MapMan graphical representation of the regulation of ATP synthesis and mitochondrial electron transport exposed to 200nM Ag^{+} for 1h at the transcriptome level. Each green or red box represents a transcript with green being down-regulated and red being up-regulated.

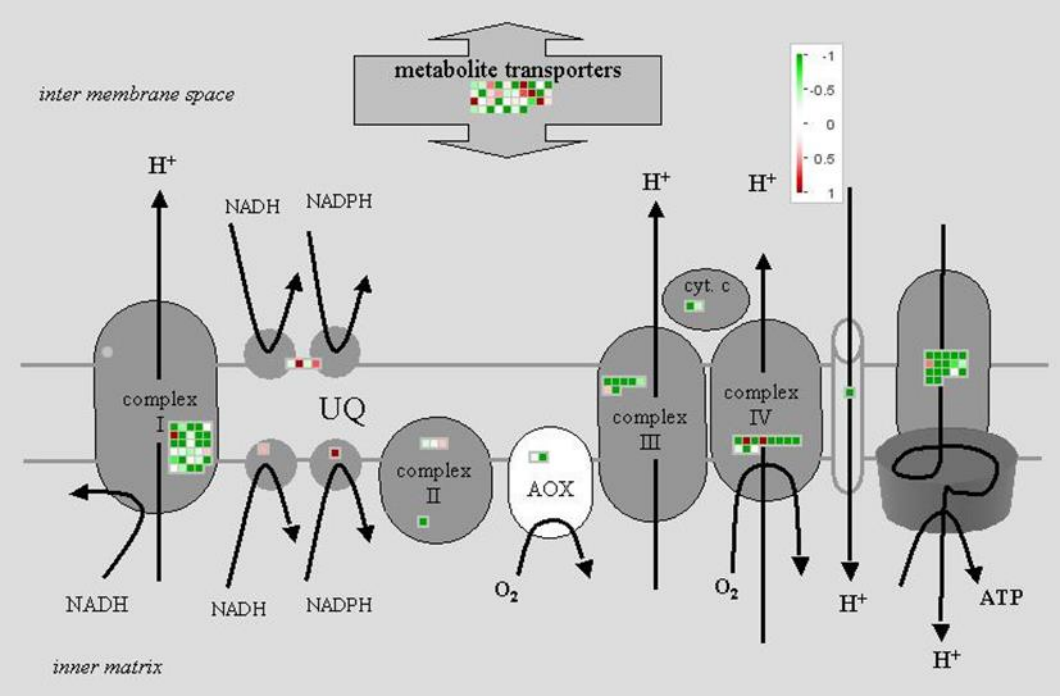


Figure S9: Quantification of lipid bodies per cell in *C. reinhardtii* exposed to Ag^{+} for 1 hour. In each exposure concentration, the algae were stained with Nile red and lipid bodies in cells in 10 microscope slide views are counted and averaged per cell.

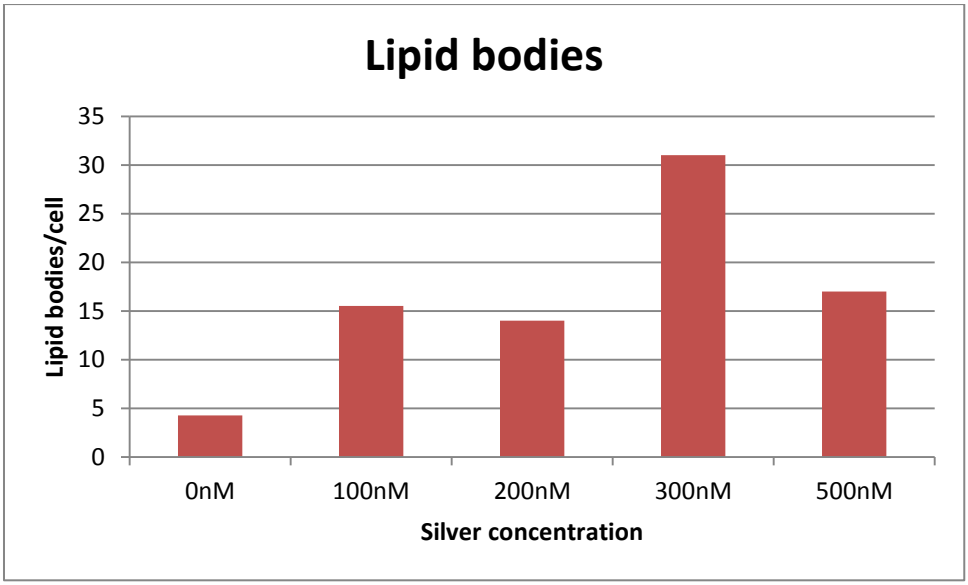


Figure S10: A graphical representation (KEGG) of the autophagy pathway. The boxes represent proteins involved in the pathway, with those in green being annotated in *C. reinhardtii*. The proteins regulated on exposure on Ag^{+} are circled in red.

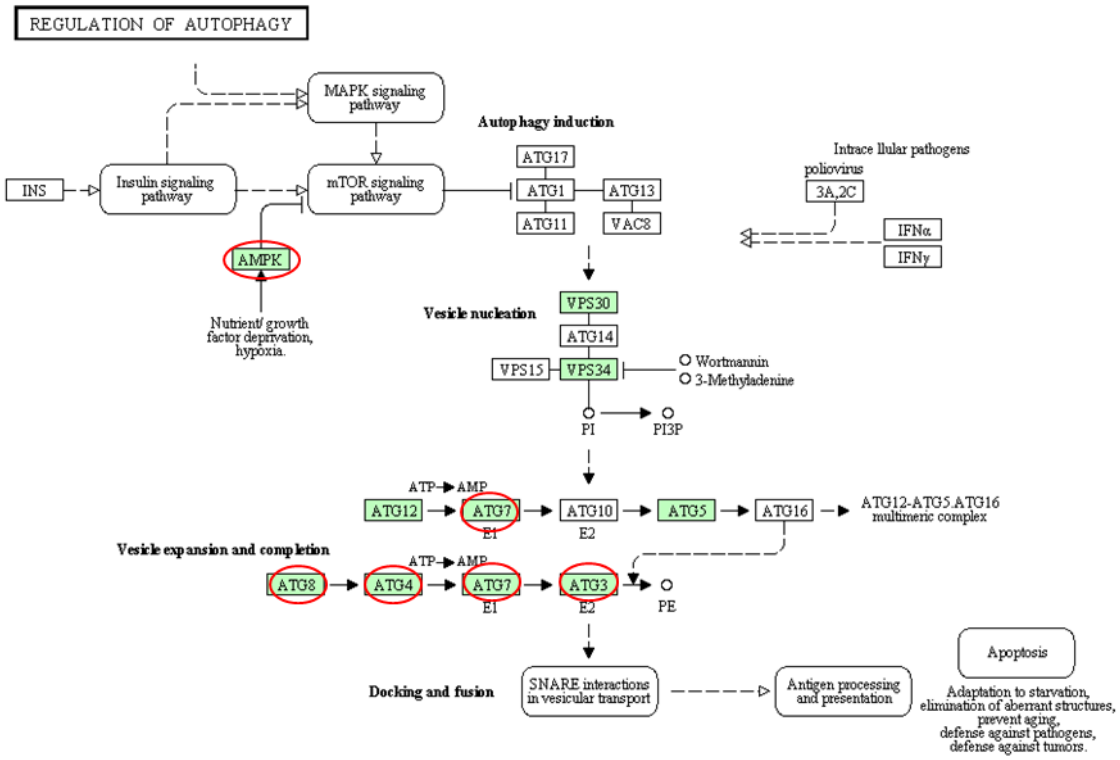


Figure S11: MapMan graphical representation of the regulation of the protein targeting in *C. reinhardtii* exposed to 200nM Ag^{+} for 1h at the transcriptome level. Each green or red box represents a transcript with green being down-regulated and red being up-regulated.

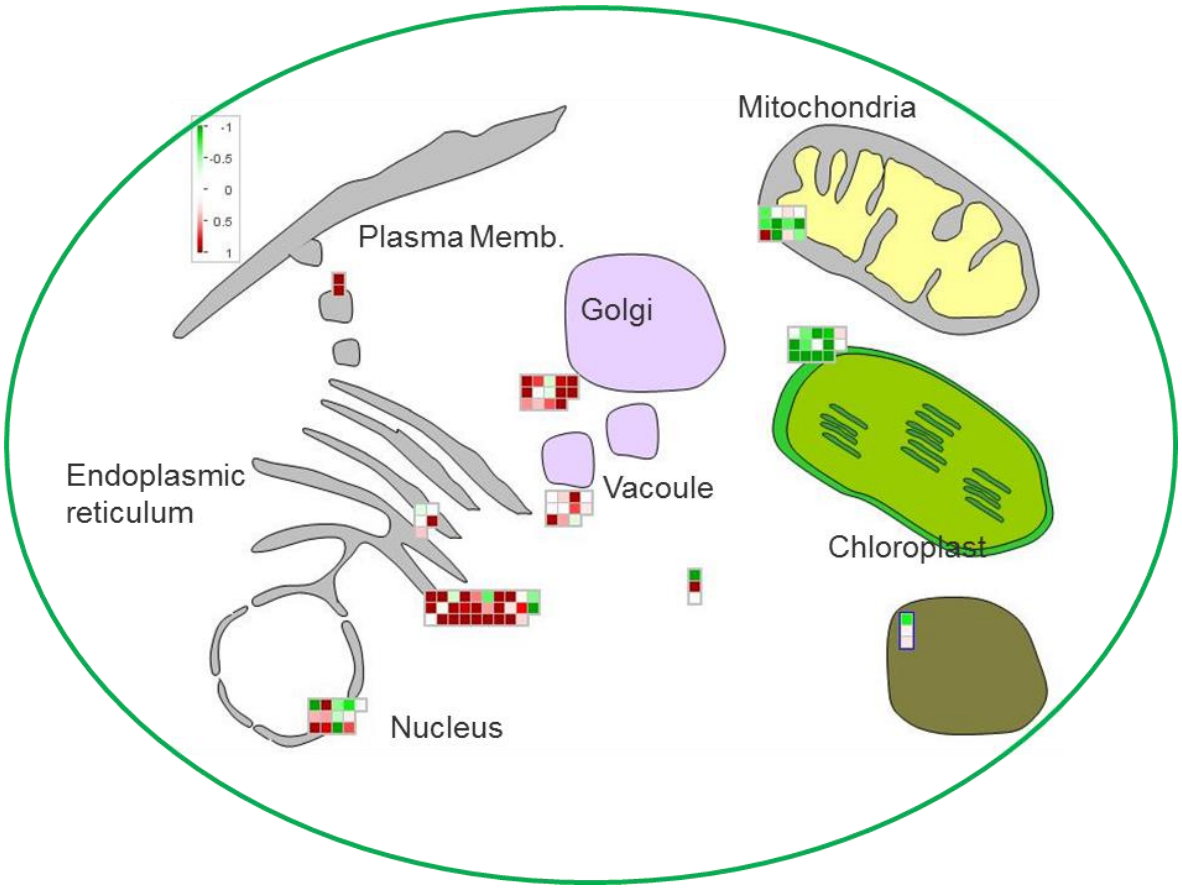
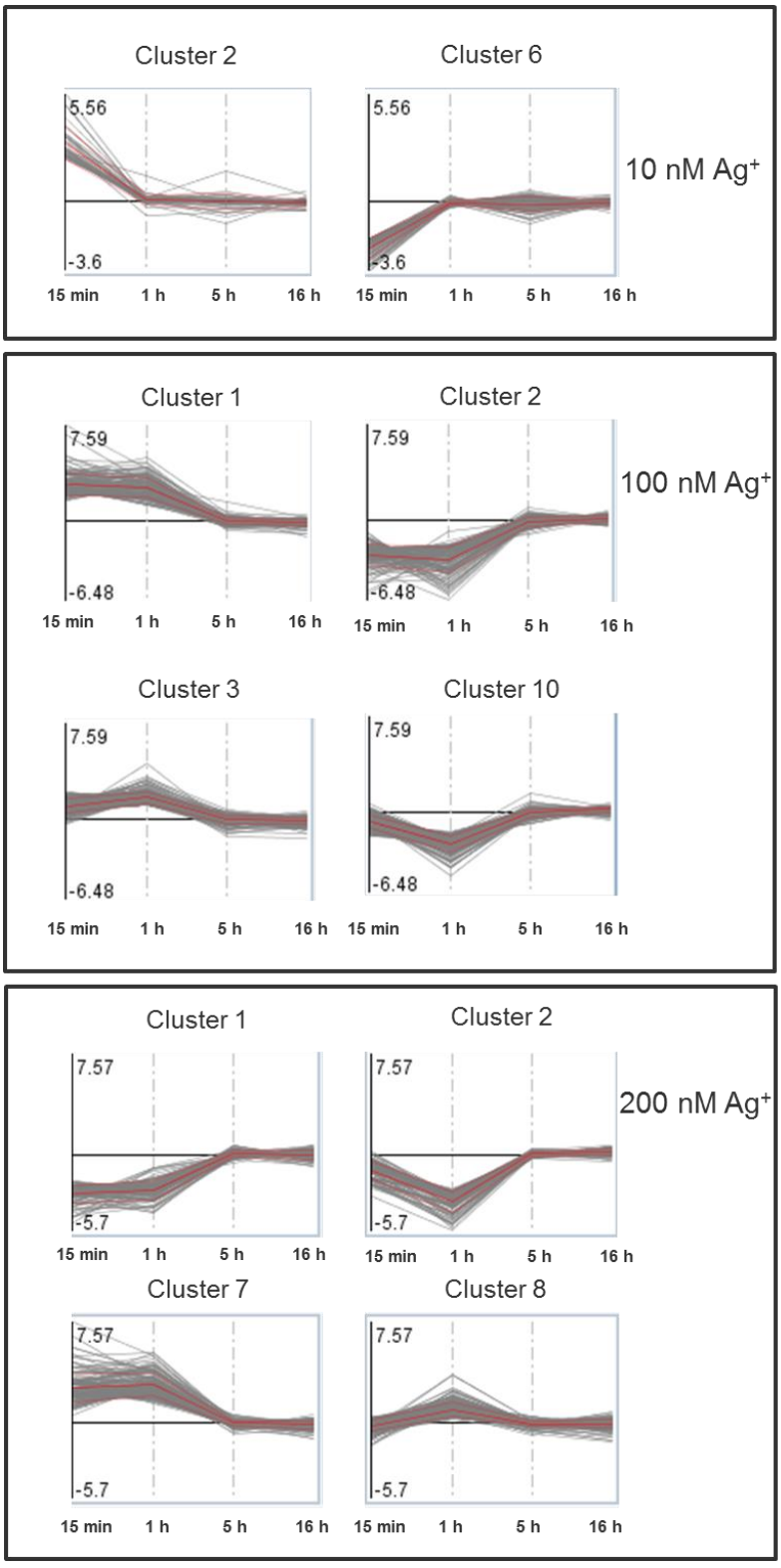


Figure S12: Eucleidean clustering of transcripts with similar expression patterns at time points of 15 min, 1 h, 5 h and 16 h in *C. reinhardtii* at concentrations of 10, 100 and 200 nM Ag^{+}. The y- axis in each graph is the log 2 fold change of abundance and the x-axis are the different time points.



1. Thimm O*, et al.* (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J* 37(6):914-939.
2. Nestler H, Groh KJ, Schonenberger R, Eggen RI, & Suter MJ (2012) Linking proteome responses with physiological and biochemical effects in herbicide-exposed *Chlamydomonas reinhardtii*. *Journal of proteomics* 75(17):5370-5385.
3. Wong JW & Cagney G (2010) An overview of label-free quantitation methods in proteomics by mass spectrometry. *Methods Mol Biol* 604:273-283.
4. Zhang B*, et al.* (2006) Detecting differential and correlated protein expression in label-free shotgun proteomics. *J Proteome Res* 5(11):2909-2918.