INTERNSHIP REPORT

Differential gene expression of *Chlamydomonas* during State1 to State2 Transitions

An internship report presented in
partial fulfillment of the requirement
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in Computational Biosciences

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Abstract

Chlamydomonas reinhardtii is a unicellular green alga that has been extensively used for research into molecular biology of photosynthesis. Its genome has been completely sequenced and microarrays have been designed so that whole genome responses to perturbations in the organism's environment can be measured. *Chlamydomonas* regulates light absorption in its two photosystems by rapidly rearranging the light absorbing proteins from PhotosystemII (called state1) to surround PhotosystemI (state2), thus delivering more light energy to PSI when the situation calls for it. State2 conditions can be induced by placing the cells in an anaerobic environment. The aim of the experiment, then, is to determine the respective patterns of gene expression of cells in State1 and in State2 acclimating cells.

RNA has been isolated from cells under both conditions, labeled with the dyes cy3 and cy5 and hybridized together to oligo nucleotide probes on the *Chlamydomonas* v2 micro array slide. Emphasis has been placed on determining the expression patterns for genes involved in photosynthesis and in potential sensing pathways. Data from repetitive and dye swapped slides has been introduced into Gene spring data analysis software for normalization, filtering, clustering genes into common expression patterns, and gene ontology.

Chlamydomonas reinhardtii's adaption to anaerobic conditions was studied using high throughput microarray technique, which provided more insights about the proteins that

are involved in the dynamic adaptation process state transitions to balance the excess energy. The transcripts encoding kinases which are responsible for the state transitions were more abundant. Increase in the levels of transcripts encoding enzymes involved in the transcription, translation and signal transduction pathways was noticed suggesting a prominent role in the process of adaptation to anaerobic conditions. Transcripts encoding photosystems and light harvesting complexes also increased suggesting a role in the state transitions. The results suggest various dynamic adaptation processes of C*halmydomonas reinhardtii* to the anaerobic conditions.

Goals of the Project

1. The goal of the project is to determine the pattern of gene expression in response to imbalances in the energy states of photosystem I and photosystem II.
2. 1 hour and 2 hours of oxygen deprivation was used to determine rapid responses in gene expression for proteins involved in acclimation of the photosynthetic apparatus to these energy imbalances which are driven by the oxygen deprivation.

Internship Details and Requirements

1. The internship mainly aims to determine the gene expression changes due to the anaerobic conditions provided.
2. High throughput Microarray technique was used to study the whole organism's genome and changes of gene expressions due to the anaerobic conditions provided.
3. This internship has given a very good exposure to molecular biology techniques and image analysis soft wares like Gene Pix.
4. It also is a very good exposure to Gene spring software which is a powerful tool for the data analysis of microarray techniques. This was used to analyze the microarray expression data.

Introduction:

Chlamydomonas reinhardtii:

Chlamydomonas reinhardtii is green algae and is a single celled organism. It belongs to kingdom Protistae. It is 10 microns in diameter and is present in soil, Oceans, fresh water and on mountain tops. *Chlamydomonas* has a cell wall, chloroplast, pyrenoid and an eyespot which can sense the light. It has two anterior flagellae which help in swimming. Previously it was believed that only plants are capable of photosynthesis but some micro organisms like algae are also capable of photosynthesis.

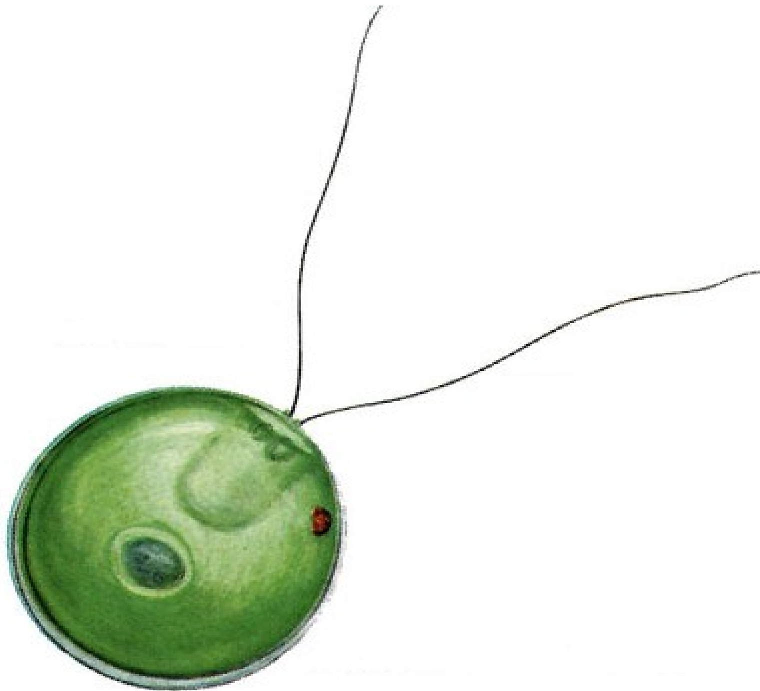


Fig1: *Chlamydomonas reinhardtii*

Source:

http://www.botany.hawaii.edu/BOT201/Algae/Bot%20201%20Chlamydomonas.jpg

Chlamydomonas has a very simple lifecycle and its genome has been elucidated. cDNA and genomic libraries of *Chlamydomonas* are available (Grossman, A.R et al., 2003). It is used as a model organism for studies of photosynthesis, responses to external stimuli, cell and molecular biology etc. *Chlamydomonas reinhardtii* is used here to study the gene expression during state transitions.

Photosynthesis and state transitions:

Plants, some bacteria and some protistans are capable of photosynthesis, a process of converting light energy into chemical energy. *Chlamydomonas reinhardttii* is a protistan which is capable of photosynthesis. Photosynthesis is mainly a two stage process. The two stages are energy transduction (light dependent reaction) and carbon assimilation (light independent reaction). Light dependent reaction is the first stage of photosynthesis where the direct light energy is used to make energy carrier molecules (ATP and NADPH) that are used in the second stage. In the second stage the products of light reaction are used to synthesize carbohydrates. In phototrophic organisms energy transduction and carbon assimilation take place in specialized organelles called chloroplasts. Chloroplasts are double membrane bound organelles and these two membranes are separated by an inter membrane space. Stroma is a gel-like matrix which is enclosed by the inner membrane.

Stroma contains enzymes for carbon, nitrogen and sulfur fixation, DNA, mRNA, and ribosomes hence the chloroplasts are known as semiautonomous organelles. Flattened sac like thylakoids are suspended inside the stroma. Single thylakoid is called as granum

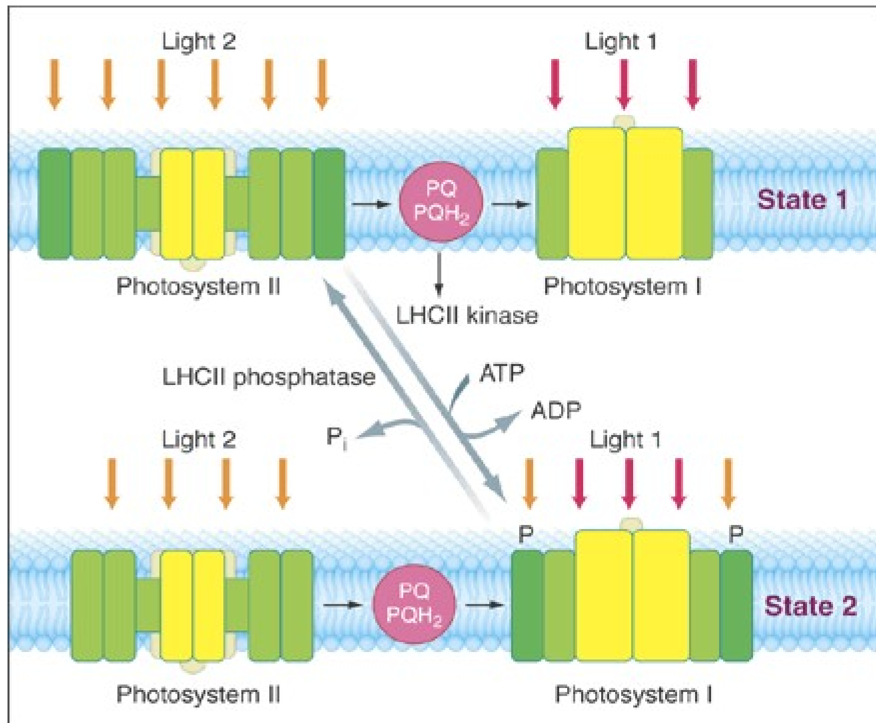
and stacks of thylakoids are called as grana. Energy transduction (light reaction) occurs in grana where as the carbon assimilation occurs in stroma.

Two types of photo systems, PhotosystemI and PhotosystemII (aka PSI and PSII), play an important role in photosynthesis. Chlorophyll molecules, accessory pigments (carotenoids and phycobilins in cyanobacteria), and associated proteins are organized into functional units called photosystems. The antenna pigments which are also known as light gathering pigments absorb photons and transfer the energy to neighboring chlorophyll molecules or accessory pigments by a process called resonance transfer until energy reaches the reaction center of photosystem.

PSI is associated with light harvesting complex I and PSII is associated with light harvesting complex II. These light harvesting complexes pass the energy they collect to the nearby photosystem; they do not contain reaction centers. Usually photosynthetic unit contains approximately 300 chlorophyll molecules (John F.Allen et al., 2003). When these chlorophyll molecules absorb energy only one chlorophyll molecule can convert this light energy into chemical energy and that is the reaction center.

Chlamydomonas regulates light absorption in its two photosystems by rapidly rearranging the light absorbing proteins between the two photosystems by state transitions. State 1 is a normal situation where the electrons are flowing equally between PSI and PSII. In state1 condition plastoquinone converts into oxidized form. This results in inactivation of kinase. Then light harvesting complex II proteins are de phosphorylated by a light

harvesting complex II phosphatase. Light harvesting complex II returns to PSII and then the energy balance is maintained (John F. Allen et al., 2003). State1 and State2 transitions are shown in the following Figure.



Source:

http://www.sciencemag.org.ezproxy1.lib.asu.edu/cgi/content/full/299/5612/1530/F1

Figure 2: State transitions)

State2 is a situation where Photosystem energy flow is unbalanced, that is where little or no electrons flow from PSI to PSII because too much was flowing from PSII to PSI. This situation can be achieved in the cells by placing the cells in the dark and anaerobic conditions. During these state transition proteins that absorb light move from PSII to PSI to reduce the flow of energy from PSII to PSI (John F. Allen et al., 2003). These proteins

are known as light harvesting proteins.

State2 condition is produced due to the excess excitation of PSII where the excess energy is transferred to PSI. When PSII is over excited more electrons enter plastoquinone due to which it becomes reduced. A thylakoid protein kinase (sst7) is activated by the reduced plastoquinone. Light harvesting complex II proteins are phosphorylated by the thylakoid protein kinase then the light harvesting complex II joins PSI to correct the imbalance of energy (Depege, N. et al., 2003).

Micro array Technique:

The Microarray technique is a very useful high-throughput technique for a variety of problems including protein: protein interactions, antibody: protein interactions, RNA copy number, DNA copy number, methylation, transcription factor binding, lectin binding and many others currently are moving from research to practical use. In the field of genetics and molecular biology the transcription of genes is important for determining a cell's fate. RNA or cDNA microarrays are used to study the expression of genes at RNA level.

In our study a Chlamydomonas expression microarray was used to study the gene expression of *Chalmydomonas reinhardttii* under anaerobic conditions. Expression analysis is a robust technique which revolutionized the field of genetics and molecular biology. In our case, we used gene expression microarrays to study the expression of genes under different cellular environments, in particular varied growth conditions and in response to different stress conditions or mutations. Gene expression of thousands of genes can be studied simultaneously using this high-throughput technique. An expression microarray consists of an orderly arrangement of genes on a standard glass microscope slide.

Individual spots contain oligonucleotides that are complementary to genes from a specific organism. These spots are approximately 50-200 microns across, depending on the array, and are arrayed in a specific order such that each spot maps to a specific gene name. The DNA chips act as templates and allow the binding of labeled cDNA or RNA to the DNA

which is bound to the glass slide. The analysis process involves extraction of RNA from the cells followed by reverses transcription of RNA to cDNA. The cDNA or an amplified RNA is fluorescently labeled and hybridized to the array. Un-base paired cDNA is removed by washing. In our study we have 2 samples which are treated with O2 and N2. The N2 cells are in state2. The control O2 cells are in state 1.

The color of each spot corresponds to the ratio of RNA of O2 and N2 treated samples which bind to the single stranded DNA of chip. By determining this ratio it is possible to determine whether the RNA for a gene has increased or decreased in amount during the state transition. An increase may mean that the gene has been induced. A decrease may mean it has been down regulated. The arrays were made at Stanford genome center. They are the chlamyV2 arrays containing two identical arrays of approximately 10000 oligonucleotides each 70 bases in the length.

Materials and methods:

Cell Culture:

Chlamydomonas reinhardtii strain CC 125 was inoculated in Cox's *Chlamydomonas* medium in conical flasks at pH 7.0 and at room temperature. Continuous illumination was provided. The conical flasks containing media were subjected to constant shaking at 150 rpm by agitating on a rotator shaker. *Chlamydomonas* cells were grown for 48hrs. As the aim of the experiment is to determine the respective patterns of gene expression of cells in State 1 and in State 2 acclimating cells, the cells were shaken in the dark for 2hrs and were bubbled with N2 for 2hrs.These two treatments result in state 1 and state 2 conditions respectively.

Isolation of RNA:

RNA isolation procedure is given below.

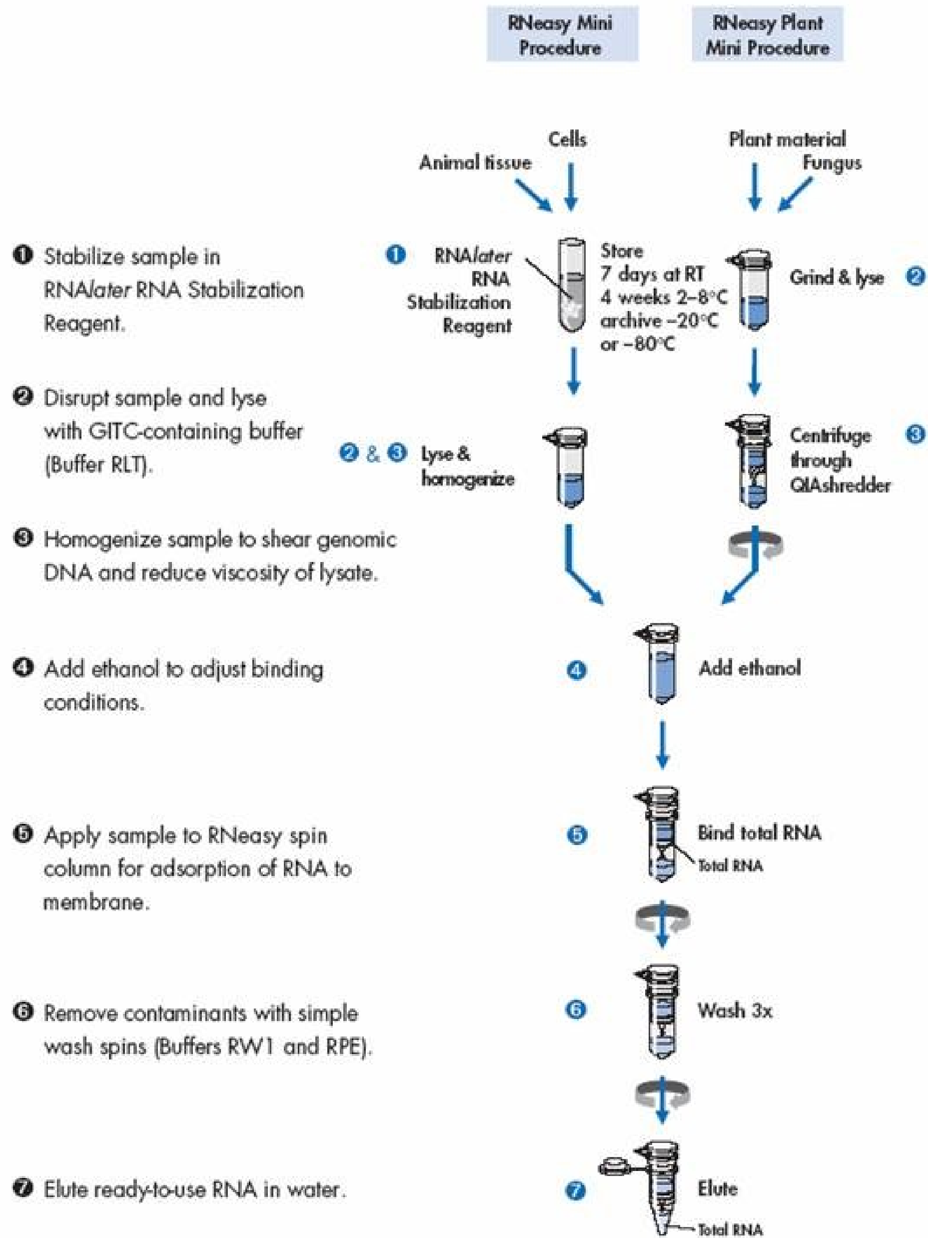
1. After subjecting the cell cultures to O2 and N2 treatments the solutions were centrifuged for 5 minutes with full speed.
2. 1ml of Trizol reagent was added to the pellets and the samples were incubated for 5 minutes which allows the dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added and tubes were shaked vigorously.
3. After shaking the tubes were incubated for 2- 3 minutes and then the samples were centrifuged at 12000 × g for 15 minutes. Due to this centrifugation the mixture separates into three phases and RNA remains in colorless upper aqueous phase.

4. The aqueous phase was transferred to a fresh tube and the RNA from aqueous phase was precipitated by using 0.5ml isopropyl alcohol.
5. The RNA pellets were washed with 1 ml of 75% ethanol. The samples were centrifuged at7, 500 × g for 5 minutes.
6. The pellets were dissolved in proper amount of DEPC treated water.

Source:http://www.invitrogen.com.ezproxy1.lib.asu.edu/site/us/en/home.reg.us.html

RNA Purification:

For each time point two RNA preparations (O2 and N2 treated) were generated from independent experiments. RNA preparations were further purified with the RNA easy Mini kit (QIAGEN) (http://www1.qiagen.com/). The procedure is as follows.



Source:http://abe.leeward.hawaii.edu/Protocols/RNeasy%20Principle_files/image002.jpg

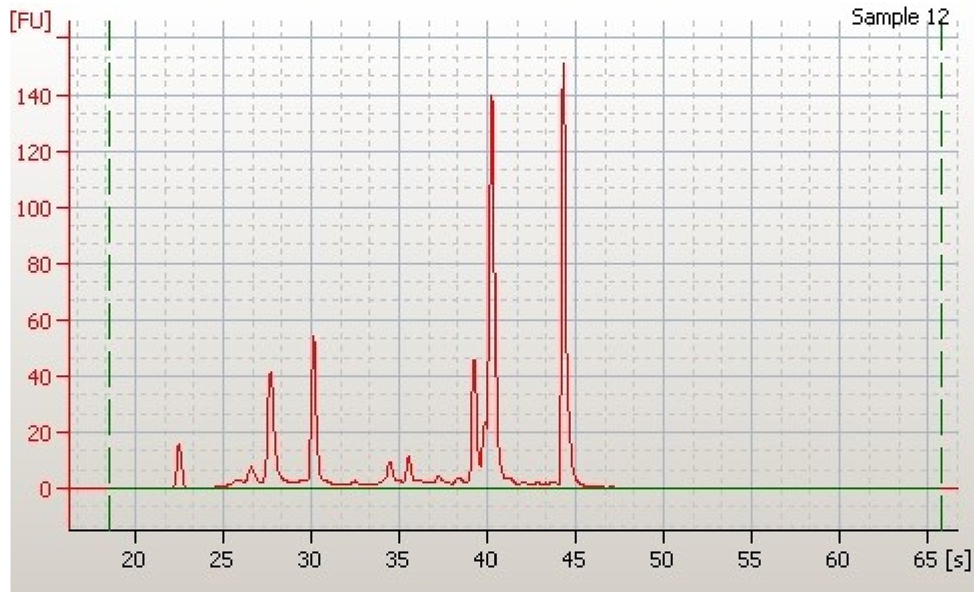
Figure 3: Protocol of RNA extraction

The procedure is as follows.

1. The sample volume was adjusted to 100µl with RNase free water then 350 µl of buffer RLT was added mixed thoughly.
2. 250 µl of ethanol was added to the diluted RNA and was mixed throughly.
3. The whole sample (700 µl) was added to an RNeasy mini spin column and centrifuged for 15 sec at 8,000 rpm.
4. After the centrifugation a new collection tube of 2 ml was taken into which the RNeasy column was transferred. After adding 500 µl of buffer RPE onto RNeasy column it was centrifuged for 15 seconds .
5. RNeasy column was placed in a new 2ml collection tube and centrifuged at full speed for 1 min.
6. RNeasy column was transferred to a new 1.5ml collection tube. 30-50 µl RNase free water was added directly onto the RNeasy silicaon gel membrane and centrifuged for 1 min at 10,000 rpm.

Quality of RNA:

RNA quality was checked by using Agilent 2100 bioanalyzer. Efficiency of labeling of RNA depends on the RNA quality and integrity. The efficiency of reverse transcription may be lowered due to the impurities. So RNA must be free of contaminants like DNA, proteins, or cellular materials. RNA integrity is another factor which is responsible for the efficient amplification of RNA and Agilent 2100 bioanalyzer is used to check the integrity of RNA.



This is a Screen capture of an Agilent 2100 Bio analyzer electropherogram of an RNA sample. The first band corresponds to the marker. The peak at 40 seconds is 18s ribosomal subunit and peak at 45 would be 28s ribosomal subunit. The remaining small peaks may be 5s and 5.8s subunits of RNA.

Amplification and Labeling:

Labeling of RNA is done by incorporating labeled nucleotide into RNA. Amino allyl labeled UTP was incorporated into the copies of RNA generated in an amplification reaction. Once incorporated into the RNA, the UTP couples to Cy3 or Cy5 dye molecules that have an NHS ester component. Cy3 and Cy5 are the fluorescent dyes belonging to the cyanine dye family. They have reactive groups through which they bind to the compounds like nucleic acids or proteins. The amplification and labeling involves the following steps. Source: (http://www.ambion.com/).

Synthesis of first Strand cDNA by using Reverse Transcription

1. 1 µl of T7 Oligo (dT) was added to the RNA and the RNA was denatured at 70ºC for 10 minutes and then placed on ice.
2. 8 µl of Reverse Transcription Master Mix (10x First Strand Buffer ,dNTP Mix,RNase inhibitor,Array Script) was added and then incubated for 2hrs.

Syntheis of the Second Strand cDNA

1. To the sample 80 µl of Second Strand Master Mix which is made up of Nuclease free waterffer,dNTP, 10x Second strand Buffer,dNTP Mix, DNA Polymerase, RNase H was added and incubated for 2 hrs at 16 ºC.

cDNA Purification

1. 250 µl of cDNA Binding bufferr was added to each sample and the mixture was passed through a cDNA filter catridge.
2. After passing cDNA Binding buffer, wash buffer of 500 µl was added to the cDNA filter catridge and cDNA was eluted 2 times with 9 µl of Nuclease free water.

Synthesis of aRNA by using In Vitro Transcription

1. 26 µl of IVT Master Mix made up of aaUTP,ATP,CTP,GTP Mix, UTP Solution,T7 10X Reaction Buffer and T7 Enzyme Mix was added to each sample and incubated for 4-14 hrs at 37 ºC.
2. After 14 hours of incubation , Nuclease-free Water of 60 µl was added to each sample.

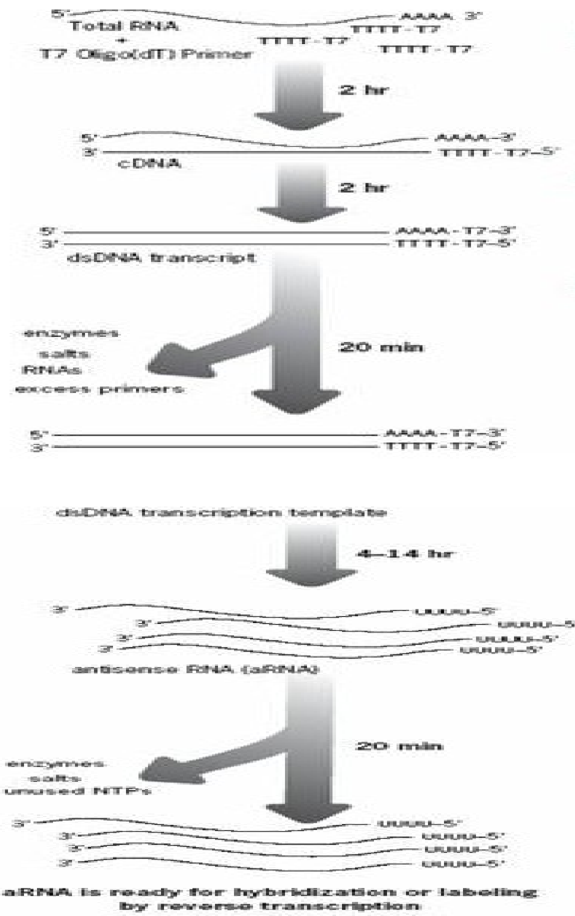
aRNA Purification and dye coupling

1. 350 µl of RNA binding buffer and 250 µl of 100% ethanol were added to each sample and passes through an aRNA filter Catridge.
2. 650 µl of wash buffer was passed through the filter catridge and aRNA was eluted with 100 µl of pre heated Nuclease free water.
3. Dyes Cy5 and Cy3 were resuspended in 11 µl of DMSO.
4. aRNA concentration was determined and 5-20 µg aRNA was vaccum dried.
5. 5 µg of aRNA is present in 16 and 17 µl of aRNA samples treated with N2 and O2 respectively.Dried aRNA was resuspended in 9 µl of Coupling buffer.
6. 11 µl of Cy5 was added to aRNA sample treated with N2 and 11 µl of Cy3 was added to O2 aRNA sample treated with O2 and both samples were incubated for 30 minutes in the dark.
7. 4.5µl of 4M Hydroxylamine was added to each sample and incubated for 15 minutes at room temperature in the dark.
8. 5.5 µl of nuclease free water was added to each sample to bring the final volume to 30 µl.

Purification of labeled aRNA

1. 105 µl of aRNA binding buffer and 75 µl of 100% ethanol were added to each sample and passed through a labeled aRNA Filter Catridge.500 µl of wash buffer was passed through the Filter Catridge.
2. After passing wash buffer pre heated 10 µl of Nuclease-free water was used to elute labeled aRNA.
3. Dye labeling was reversed with Cy5 to O2 and Cy3 to N2 in a separate

experiment and all experiments were repeated with independly isolated RNA samples.



Source:http://www.rockefeller.edu/genomics/pdf/Ambion_aRNA_Amplification_Manual_Affymtrix.pdf

Fig 4: Amplification of RNA

Hybridization

1. Arrays were prehybridized in 3X SSC-0.1% SDS-10mg/ml bovine serum albumin for 20 minutes at 50 ºC.

2. The slides were immersed into ddH20 and isopropanol for 2 minutes.
3. The slides were dried by centrifuging them for 5 minutes.
4. For hybridization we have used 48 pico moles of labeled target that is 2 µl each of N2 and O2 treated labelled aRNA is used.
5. 60 µl of sample (2µl each of N2 and O2 treated labelled aRNA,30 µl of 2X hybridization buffer, and 26 µl of RNase free H2O) was loaded to a prehybridized microarray and was hybridized at 50 ºC for 16 hours by placing in hybridization chamber.
6. After hybridization the slides were washed for 5 minutes in 2XSSC,0.1%SDS, then slides were washed for 5 minutes in 1XSSC and finally the slides were washed for 5 minutes in 0.05XSSC solution.
7. The slides were dried by centrifuging them for 5 minutes.
8. Agilent scanner was used to scan the arrays and it is compatible to Cy3 and Cy5 dyes.

   Source: (Kate Rubins/Brown Lab 2006)

Data analysis

1. The raw images obtained by scanning of array are stored as TIF files which later are transferred to feature extraction software like Genepix 8.0 . (http://www.moleculardevices.com/)
2. Gene Pix is an image analysis software which can import TIF imges for further analysis. The images generated by the scanner are to be quantified which is done by Gene Pix Pro software. It quanitifies the spot intensity by considering the background noise as well.
3. Gene Pix is a feature extraction software which can extract data from the scanned images. A predefined microarray layout is used to define the position of spots and those spots that are distorted due to dust or high background were not included in the analysis.
4. The results of Gene Pix were displayed in Gene pix results (GPR) files. It uses the images file (TIF) genrated by scanner and convert it into text file called GPR file.
5. An inbuilt algorithm of Gene Pix converts the shape of spot and intensity of pixel into signal.
6. It is an efficient image analysis software which calculates upto 108 measurements for each spot which includes mean, median, ratio of mean, ratio of median, standard deviations etc.
7. Once the array had scanned and quantified then the data should be noramlized.The data was further normalized and filtered by using Genespring software. It is a powerful tool to analyse the microarray data .
8. Main aim of expression analysis is to find out the expression of genes of our

interest. But the expression may be sometimes due to the experimental errors which should be corrected to get the biological gene expression with out an error.

9. Normalization methods help by reducing the experimental errors and give only biological gene expression. There are different normalization methods available in the genespring software. Lowness normalizayion method was used to normalize the data as the experiment is two colur experiment.
10. Once the data is normalized it can be analyzed by using various statistical tools that are provided by Genespring.
11. This study mainly intends to find the possible proteins that are involved in the process of state transitions.
12. The features of Genesprig software allow us to find out the interesting genes and their expression.
13. Different approaches that are used to analyze gene expression data as follows.

Fold changes

Fold changes were used to find out the over expressed and under expressed genes due to the anaerobic conditions provided. Filter on fold change feature of Genespring was used to filter the genes basing on their fold changes. Fold change is nothing but the average expression of control(Cy3) over treated(Cy5) in all samples. Fold changes are found for 2 conditions (1 hour and 2hour nitrogen treatment) to compare the gene expression in both conditions.The results obtained by using filter on fold changes were disscused in the results section.

Clustering

Generically clustering is a multivariate non statistical process used to group data into subsets that reflect some common, measurable trait. For gene expression, clustering uses a defined measure (in this case, Euclidean distance) to partition the expression values into subsets that should reflect either co-expression or common expression patterns. Co-expression assumes a common transcription factor or regulatory pathway while common expression makes no such biological assumption. Different clustering algorithms are present in the GeneSpring 7.2.1 software (Agilent corporation, Palo Alto, CA) which can be used to further distinguish groups of gene expression patterns. Quality threshold clustering is used to group the genes into a number of clusters based on their relative quality metric, a value assigned to expression datapoints based on their subjective reliability.

It is known as quality threshold as dissimilar genes were not present in the cluster and hence ensures good quality cluster. The gene list which is formed based on the filter of fold change is clustered by using QT cluestering. The QT clustering is formed based on an inbuilt algorithm which selects a random gene and finds a gene which has greatest similarity to that selected gene and adds it to the cluster. This process continues until no gene can be added to the cluster. Then a second gene will be selected randomly and forms a seed for new cluster. Like this the process continues and the genes which can not be classified forms as an unclassified group. Once the QT clusters are formed, genes which come into a cluster have similar expression profiles and may be they have some similar biochemical features in common, the expression of the genes that come under the same

cluster may be controlled by a similar mechanism. They may have coexpression due to some common biochemical factors. Previous studies proved the significance of hydrogenase gene in anerobic conditions (Mus,F et al., 2007). The hydrogenase was supposed to be expressed during anaerobic conditions and it was found up regulated.This gene has been used as a control gene to ensure that our cells are functioning as would be expected of cells under anaerobic conditions. The genes that were clustered along with the hydrogenase gene were found. These genes may have some functional similarities with the target gene (hydrogenase). Next appoarch was to explore gene ontology feature of the genespring software.

Gene ontology

An important part of gene expression data analysis is finding the biological context of the differentially expressed genes. Genespring has a feature called Gene Ontology (aka GO, www.geneontology.org) which provides information about molecular identities, cellular mechanisms, subcellular location and biochemical pathways. The aim of this experiment is to find out the expression of genes which are involved in the state transitions. The gene ontology helps us to find out the biological proceses and pathways that are effected due to the anaerobic conditions provided. By using the gene ontology feature various gene lists were generated which include photosynthetic pathway genes, biochemical and metabolic pathway genes .

Results

Results are based on differential expression of genes which provides the context through which to make assumptions about functions in the cell. No protein levels were measured; only projected levels through the proxy of transcript copy number were inferred. However, it has been shown that network and pathway analysis of gene expression correlates well with observed phenotypes, so we propose that the analysis of gene expression is a good indicator of cellular functions.

Oligonucleotide based Microarray has used to find the whole genome response to the anaerobic conditions. The genome response to anaerobic conditions was monitored by measuring the abundance of transcripts. As the cells were subjected to Nitrogen treatment which is not a very favorable condition for the cells, many transcripts were less abundant as expected. But interestingly some genes are up regulated suggesting a prominent role in the state transitions and acclimation to anaerobic conditions. [2-fold is arbitrary but has been used historically to indicate a non-statistical but convincing change in transcript amounts. It also is a single numerical digit on a log2 chart] Many transcripts had shown a change of 2 fold up or down. 6 slides were done per each time point 3 regular and 3 dye swap. The fold change we get is the mean of the median. The fold changes were further confirmed by manually checking those spots in Gene Pix .The 2 hour up regulated genes were shown in table 1 , 2 hour down regulated genes were shown in table2, 1 hour up regulated genes were shown in table 3 and 1 hour down regulated genes were shown in table 4. The transcripts which had shown significant change were further studied.

Kinases

This study intends to find possible proteins which are involved in state transitions. Kinase plays an important role in the state transition (Depege, N et al., 2003). Kinase phosphorylates the light harvesting complex protein II which joins PSI to absorb the excess energy. The kinases are believed to play significant roles in the process of state transitions by correcting the energy imbalances which are caused due to anaerobic conditions. The transcripts of various kinases had shown more than 2 fold change up regulation suggesting a prominent role in the process of state transitions. Increase of transcripts of kinases also suggests a possible role in some useful signal transduction cascades. The probable serine/threonine kinase which was found to be up regulated may have a role in state transitions. The fold changes of the genes encoding kinases due to 2hour and 1 hour nitrogen treatment were shown in the table number 5 and 6 respectively.

Photosynthesis genes

The abundance of transcripts encoding proteins of photosynthetic process declined and there is more decline in the transcripts which were subjected to 2 hours of nitrogen treatment than 1 hour of nitrogen treatment. Most of the photosynthetic genes were down regulated. This may be because of the stress due to the anaerobic conditions whereas, some photosynthetic genes which are up regulated suggesting a prominent role in state transitions. The photosynthetic genes of 2 hour and 1 hour nitrogen treatment were shown in table 7 and 8 respectively.

Stress genes

The transcripts of genes encoding chaperones are noticed in both 2hour and 1hour nitrogen treatment. Chaperones are the proteins which are induced in the conditions of stress (Zhang, Z et al., 2004) As the cells were subjected to stress the transcripts encoding heat shock proteins were up regulated. Heat shock proteins bind to the proteins and stabilize them during the protein folding, degradation and transport. These proteins are expressed to counter attack the unfavorable stress conditions created due to the nitrogen treatment. The heat shock proteins are classified based on their molecular weight for example HSP22, HSP70 etc. Increase in the transcripts of chloroplast heat shock protein 22 is may be due to the organelle's (chloroplast) response due to nitrogen treatment. The stress related proteins that are up regulated were given in the table 9.

Hydrogenase

Previous studies had proved the ability of *Chlamydomonas reinhardttii* to produce hydrogen during anaerobic conditions (Mus, F et al., 2007). The hydrogenase gene is responsible for the production of hydrogen by combing the electrons of electron transport chain with protons during anaerobic condition. Various hydrogenases like geranyl hydrogenase, Fe-hydrogenase precursor, iron-hydrogenase HydA2 had shown more than 2 fold up regulation due to anaerobic conditions provided in this experiment. This increase in hydA2 mRNA, which is only detectable in the absence of oxygen, indicates that our cells were indeed subjected to anaerobic conditions during treatment.

Metabolic pathway genes

Transcripts of genes involved in the protein metabolism like transcriptional, translational and post translational regulatory elements were up regulated suggesting a need to control the translational and post translational events in the chloroplast as the organism is exposed to stress conditions like anaerobic conditions by treating with nitrogen. The significant up regulation of transcripts of genes encoding proteins (enzymes) of translation and post translational modifications suggests that these proteins are involved in the process of translation and post translational modifications that occur in the chloroplast (Zhang, Z et al., 2004). May be these proteins help in the structural or functional changes that occur in chloroplast due to anaerobic conditions. The transcript abundance of protein metabolism genes was shown in the table number 10.

Some of the genes of DNA metabolism were up-regulated which may not suggest any particular significance in this situation as they are the regular Topoisomerases, and DNA polymerases. But some of the electron transport genes are also up regulated suggesting a role in state transitions and acclimatization to the anaerobic conditions. Anaerobic conditions affect the electron transport chain by reducing plastoquinone which participates in the downstream events which finally result in the transfer of light harvesting complex proteins from PSII to PSI in order to balance the excess energy at PSI. The fold changes of genes encoding electron transport chain proteins of 2 hour and 1 hour nitrogen treatment were shown in the table number 11 and 12 respectively. Genes coding various signal transduction proteins are up regulated suggesting a significant role of the signal transduction proteins in the process of acclimatization to anaerobic

conditions. Signal transduction pathways are induced due to external or internal signals (Zhang, Z et al., 2004). As the cells were treated with nitrogen which is an alteration from regular environment, signal transduction genes are expressed to in response to the change in the external environment.

Hypothetical proteins

Transcripts of genes of hypothetical proteins increased due to the nitrogen treatment. They had shown more than 2 fold increase in their abundance suggesting a prominent role in the acclimatization process to the anaerobic conditions. These results suggest that still lot of research has to be done to find out the functions of these hypothetical proteins. The transcript abundance of hypothetical proteins of 2 hour and 1 hour nitrogen treatment were shown in the table number 12 and 13 respectively.

Conclusions

The analysis of gene expression of an organism which is exposed to unfavorable environmental conditions by using high throughput microarray is a great advance in the scientific field. The microarray technique was used to explore the state transitions that occur due to anaerobic conditions and the proteins that are involved in the state transitions process.

The cells were treated with nitrogen for 1hour and 2hours respectively in two separate experiments to provide anaerobic conditions to the cells which result in the state transitions. Comparative gene expression analysis of *chlamydomonas reinhardtii* was done to find out the effects of the anaerobic conditions due to the nitrogen treatment.

Most of the photosynthetic genes were down regulated except some genes which are involved in the process of state transitions which include photo system II core complex protein, Light harvesting proteins etc. The light harvesting chlorophyll a/b complex (LHC-II) found over expressed 3.25 fold. It regulates energy distribution between the two photosystems.

Kinases which play a significant role in the state transitions were up regulated due to the nitrogen treatment. A probable Serine threonine kinase is over expressed 4.351 fold. Serine threonine kinase activity is responsible for LHC II phosphorylation.

As the cells were subjected to stress conditions the genes encoding for heat shock proteins and chaperones were expressed which play a significant role in protein folding and stability. Chaperone protein dnaK (Heat shock protein 70) is over expressed 3.116 fold and has functions in refolding of misfolded proteins that are essential under stress.

Many metabolic genes were over expressed that include those involved protein synthesis, DNA metabolism, electron transport etc suggesting a prominent role in the acclimatization of *Chlamydomonas reinhardttii* to anaerobic conditions. Tbc2 is 2.741 fold up regulated. Tbc2 is involved in the translation of the chloroplast *psbC* mRNA of the eukaryotic green alga *Chlamydomonas reinhardtii.* PsbC encodes polypeptides of PSII.

Many hypothetical genes of unknown function are over expressed which suggest a prominent role in the process of acclimatization of cells to anaerobic conditions. *Chlamydomonas reinhardtii* is an excellent model organism for the research, it has dynamic adaptation mechanisms to stress conditions one of which was explored a little to know the organism's dynamic ability to acclimatize to stress conditions.

Future directions

The present research was mainly focused on the biological significance of the treatment and interesting proteins that are responsible for the dynamic adaptation process in green alga *chlamydomonas reinhardttii.* The future goals will be to do more time points and find out which time point has great significance. Statistically significant genes were found using gene spring software but the future will be to do in depth statistical analysis on the gene expression data by comparing multiple time points. Other future work would be to do a peptide array by subjecting the *Chlamydomonas reinhardtii* to anaerobic conditions and then to compare the genome with the proteome.

Appendix: Tables

Table 1

2 hour up regulated genes

Fold change	Genbank Description
3.023	(-) flagellar autotomy protein Fa1p
2.017	(+) Cytochrome B6-F complex subunit petO, chloroplast precursor
2.264	(-) protein kinase,
4.841	(-) ribose-5-phosphate isomerase
4.605	(-) Porphobilinogen deaminase, chloroplast precursor
4.459	(-) 50S RIBOSOMAL PROTEIN L13, CHLOROPLAST PRECURSOR
4.22	(-) Uroporphyrinogen decarboxylase, chloroplast precursor
4.152	(-) CHLOROPLAST 50S RIBOSOMAL PROTEIN L21
4.095	(+) Tbc2 translation factor, chloroplast precursor
3.83	(-) Ribulose-phosphate 3-epimerase, chloroplast precursor
3.728	(+) ribosomal protein 30S subunit
3.652	(-) Photosystem I reaction center subunit XI
3.507	(-) putative chloroplast 50S ribosomal protein L6
3.41	(-) rpl12 50S ribosomal protein L12
3.359	(+) light-harvesting chlorophyll-a/b binding protein Lhcb4
3.301	(+) 50S RIBOSOMAL PROTEIN L15
3.274	(-) rpl28 50S ribosomal protein L12
3.189	(-) Photosystem I reaction center subunit IV
3.088	(-) chlorophyll a/b-binding protein

Table 2

2 hour down regulated genes

Fold Change	GenbankDescription
0.0849	(-) FRUCTOSE-BISPHOSPHATE ALDOLASE 1
0.277	(+) (P13709) Female sterile homeotic protein
0.0895	(-) ATP synthase epsilon chain
0.201	(+) (Q94AU2) 25.3 kDa vesicle transport protein
0.0352	(-) Photosystem II P680 chlorophyll A apoprotein
0.223	(+) adenylyl cyclase associated protein
0.402	(-) similar to Na+/H+-exchanging protein
0.469	(-) chlorophyll antenna size regulatory protein
0.369	(+) phenylalanine ammonia-lyase
0.0207	(+) similarity to ADP/ATP carrier proteins
0.277	(+) (P13709) Female sterile homeotic protein
0.182	(+) Axoneme-associated protein
0.436	(-) (Q9C7F5) Nuclear transport factor 2 (NTF-2)
0.476	(+) (Q9C9S6) Kinesin-related protein
0.44	(-) FLJ13848 hypothetical protein FLJ13848
0.402	(+) (P13709) Female sterile homeotic protein
0.0849	(-) FRUCTOSE-BISPHOSPHATE ALDOLASE 1, CHLOROPLAST PRECU
0.0895	(-) ATP synthase epsilon chain
0.201	(+) vesicle transport protein

Table 3

1 hr up regulated genes

Fold Change	Genbank Description
3.276	(-) Ribulose-phosphate 3-epimerase
6.633	(-) protein kinase
6.207	(-) putative chloroplast 50S ribosomal protein L6
24.49	(+) light-harvesting chlorophyll-a/b binding protein Lhcb4
3.304	(+) (Q9LJK1) Glycoprotein 3-alpha-L-fucosyltransferase A
2.139	(-) (Q9VT98) CG32062 protein
3.449	(-) proline-rich protein
4.163	(-) chlorophyll a/b-binding protein
3.761	(+) (Q9SAI7) F23A5.6 protein
2.794	(-) EBNA-1 nuclear protein [Human herpesvirus 4
2.051	(-) TGFBI transforming growth factor
2.927	(-) Tax_Id=10090 Ensembl_locations(Chr-bp)
2.838	(+) (O64765) Probable UDP-N-acetylglucosamine pyrophosphorylase
6.367	(-) glutamate/malate translocator
3.189	(-) EYE2 [Chlamydomonas reinhardtii]
2.532	(+) (Q9VY72) CG32611 protein
2.046	(-) (Q9W3W9) CG3168 protein
6.128	(-) CALK protein
2.563	(-) Antifreeze glycopeptide polyprotein precursor (AFGP polyprotein)
2.55	(-) (Q9LZU1) Hypothetical

Table 4

1 hr down regulated genes

Fold Change	Genbank Description
0.0772	(+) light harvesting complex I protein precursor
0.164	(-) FRUCTOSE-BISPHOSPHATE ALDOLASE 1
0.178	(+) 60S ribosomal protein L10a
0.465	(-) chlorophyll a/b-binding protein type III precursor
0.481	(-) F3N23.26 unknown protein
0.00534	(+) MUC2 mucin 2
0.302	(+) (Q9SB76) Hypothetical 31.9 kDa protein
0.211	(-) COG2319: FOG: WD40 repeat
0.461	(+) (Q42396) Calcium-dependent protein kinase
0.317	(-) (Q9C6K5) Sm-like protein
0.45	(+) Adenylate cyclase
0.103	(-) putative arm repeat containing protein
0.315	(+) (Q9LQ04) F16P17.17 protein
0.376	(+) pyrophosphate--fructose-6-phosphate-1-phosphotransferase
0.387	(-) putative pre-tRNA/pre-rRNA processing protein
0.352	(-) Plasma membrane calcium-transporting ATPase 3
0.361	(+) (Q9SSD2) F18B13.15 protein

Table 5

Kinases 1 hour

Fold change	Genbank description
6.633	(-) protein kinase
2.716	(+) (O82514) Adenylate kinase 1
4.351	(+) (P43293) Probable serine/threonine-protein kinase
4.726	(+) (Q9FKS0) Uridine kinase-like protein
7.557	(+) putative calcium-dependent protein kinase
6.744	(+) phosphatidylinositol 3-kinase
4.382	(+) CDKN1C cyclin-dependent kinase inhibitor
3.422	(+) (P74745) Probable serine/threonine-protein kinase
4.435	(-) NIMA-related kinase 4

Table 6

Kinases 2 hour

Fold change	Genbank description
6.972	(-) calcium-dependent protein kinase
6.136	(+) phosphatidylinositol 3-kinase
5.974	(+) putative calcium-dependent protein kinase
4.266	(+) calmodulin domain protein kinase 1
4.245	(+) cAMP-dependent protein kinase regulatory chain
4.063	(+) NIMA-related kinase 3
3.882	(-) LOC234878 similar to mitogen-activated protein kinase
3.27	(+) (Q9FKG5) Similarity to receptor-like protein kinase
3.207	(+) (Q9FGB1) Protein kinase
3.033	(+) protein kinase Fa2

Table 7

2 hr photosynthesis

Foldchange	Genebank description
4.753	(-) Chloroplast30SribosomalproteinS10
4.678	(-) Porphobilinogen deaminase,"
4.324	(-) 50S RIBOSOMAL PROTEIN L13
4.322	(+) Delta-aminolevulinic acid dehydratase
4.166	(-) CHLOROPLAST 50S RIBOSOMAL PROTEIN L21
3.952	(-) PhotosystemIreactioncentersubunitXI
3.784	(-) Ribulose-phosphate 3-epimerase
3.496	(+) 50S RIBOSOMAL PROTEIN L15
3.398	(-) Photosystem I reactioncentersubunitIV chloroplast precursor(PSI-E)
3.391	(-) putativechloroplastinnerenvelopeprotein
2.284	(+) (P51818) Heat shock protein 81-3 (HSP81-3)
0.342	(-) PHOTOSYSTEM I REACTION CENTRE SUBUNIT III PRECURSOR
0.145	(+) Photosystem I reaction center subunit II
0.138	(+) light harvesting complex I protein precursor
0.0881	(-) Photosystem II P680 chlorophyll A apoprotein
0.0239	(-) chlorophyll a/b-binding protein type III precursor
0.0772	(+) light harvesting complex I protein precursor
0.178	(+) 60S ribosomal protein L10a
0.465	(-) chlorophyll a/b-binding protein type III precursor
0.352	(-) Plasma membrane calcium-transporting ATPase 3 (PMCA3)
0.401	(+) FLAGELLAR RADIAL SPOKE PROTEIN 6
0.422	(-) (Q9LIC2) Multispanning membrane protein-like
0.069	(-) outer arm dynein light chain
0.415	(+) putative blue light receptor
0.102	(+) putative ultraviolet-B-repressible protein
0.121	(-) ribosomal protein L19
0.355	(+) Glutathione synthetase, chloroplast precursor
0.285	(+) Vacuolar ATP synthase catalytic subunit A isoform 1
0.387	(-) PHOTOSYSTEM I REACTION CENTRE SUBUNIT III PRECURSOR
0.182	(+) plastidic ATP/ADP transporter

Table 8

1 hr photosynthesis

Foldchange	Genebank description
3.352	(-) putative chloroplast50SribosomalproteinL6
3.258	(+) light-harvesting chlorophyll-a/b
2.974	(+) light harvesting complex a protein
2.972	(-) chlorophyll a/b-binding protein
2.946	(+) Sedoheptulose-1,7-bisphosphatase
2.794	(+) light-harvesting complex I protein
2.692	(+) Photosystem I reaction center subunit VI
2.653	(+) Ribosome recycling factor
2.643	(-) (Q9ASS6) Peptidyl-prolyl cis-trans isomerase TLP20
2.366	(+) CHLOROPLAST HEAT SHOCK 22 KD PROTEIN
0.041	(-) 60S RIBOSOMAL PROTEIN L6 (YL16-LIKE)
0.306	(+) 30S Ribosomal protein S21
0.145	(-) (P49688) 40S ribosomal protein S2
0.368	(+) 40S RIBOSOMAL PROTEIN S5 [Cicer arietinum], 96.4% id
0.0849	(-) FRUCTOSE-BISPHOSPHATE ALDOLASE 1, CHLOROPLAST PRECURSOR
0.277	(+) (P13709) Female sterile homeotic protein
0.13	(+) probe 6 protein
0.0895	(-) ATP synthase epsilon chain
0.201	(+) (Q94AU2) 25.3 kDa vesicle transport protein
0.0352	(-) Photosystem II P680 chlorophyll A apoprotein
0.223	(+) adenylyl cyclase associated protein
0.163	(-) unknown protein [Oryza sativa
0.402	(-) similar to Na+/H+-exchanging protein
0.469	(-) chlorophyll antenna size regulatory protein
0.424	(+) hypothetical protein
0.13	(+) probe 6 protein
0.369	(+) phenylalanine ammonia-lyase
0.0207	(+) similarity to ADP/ATP carrier proteins
0.235	(+) pherophorin-dz1 protein
0.327	(+) unnamed protein product
0.277	(+) (Q9VPL9) CG3696 protein
0.358	(+) (P13709) Female sterile homeotic protein
0.486	(+) COG3670: Lignostilbene-alpha,beta-dioxygenase and related enzymes
0.445	(-) putative PKCq-interacting protein
0.474	(-) White protein
0.498	(+) (Q9V5M1) CG17765 protein (GH27120P)
0.3	(-) (Q9W3W9) CG3168 protein

Table 9

Stress genes 2 hour

Foldchange	Genbank description
3.116	(-) Chaperone protein dnaK (Heat shock protein 70)
2.366	(+) CHLOROPLAST HEAT SHOCK 22 KD PROTEIN
2.284	(+) (P51818) Heat shock protein 81-3 (HSP81-3)

Table 10

Stress genes 1hour

Foldchange	Genbank description
2.843	(-) Tax_Id=9606 Heat shock 70 kDa protein 12A
5.591	(+) CHLOROPLAST HEAT SHOCK 22 KD PROTEIN

Table 11

Protein metabolism 2hr

Foldchange	Genbank description
34.45	(-) eukaryotic translation initiation factor 5
2.053	(-) Tbc2 translation factor
3.044	(+) (O49415) Hypothetical protein thyltransferase
2.741	(+) Tbc2 translation factor, chloroplast precursor
10.21	(+) Carotene biosynthesis-related protein CBR, chloroplast precursor
2.19	(-) (O82392) Putative thiamin biosynthesis protein
2.118	(-) Translation initiation factor eIF-2B gamma subunit
2.233	(-) (O82768) Histidine biosynthesis bifunctional protein hisIE,chloroplast precursorl
2.261	(+) Tax_Id=10090 Ensembl_locations(Chr-bp):None Eukaryotic translation initiation factor

Table 12

Protein metabolism 1hr

Foldchange	Genbank description
2.24	(+) Carotene biosynthesis-related protein CBR, chloroplast precursor
23.5	(+) Tbc2 translation factor, chloroplast precursor [Chlamydomonas 1% id
3.46	(+) Probable menaquinone biosynthesis methyltransferase
9.886	(+) Tbc2 translation factor, chloroplast precursor
4.187	(-) putative thiamine biosynthesis protein
2.591	(-) Tbc2 translation factor, chloroplast precursor
2.017	(+) Tbc2 translation factor, chloroplast precursor
4.861	(+) Eukaryotic translation initiation factor 3 subunit 11

Table 13

Electron transport chain 2hour

Fold Change	Genbank Description
2.115	(+) (Q9SFB0) Putative integral membrane protein
12.96	(+) putative NADH dehydrogenase subunit 9 precursor
2.24	(+) (Q9SF45) Putative oxidoreductase
3.029	(+) putative plasma membrane-type proton ATPase
17.6	(-) 6-phosphogluconate dehydrogenase
3.592	(+) Glutathione reductase, chloroplast precursor
2.106	(+) sulfite reductase
5.642	(+) putative glyoxal oxidase
3.878	(+) probable malate dehydrogenase
20.89	(+) COG1252: NADH dehydrogenase, FAD-containing subunit
8.322	(+) (Q9LSD0) Ribonucleotide reductase
3.712	(-) PRODH proline dehydrogenase

Table 14

Electron transport chain 1 hour

Fold Change	Genbank Description
2.796	(-) (O80874) Putative NADH dehydrogenase
2.403	(+) sulfite reductase ATPase, aminophospholipid transporter
28.52	(-) Atp8a2, Ib, Atpc1b-pending
121.4	(-) (Homoserine dehydrogenase) (HDH)
10.34	(+) (O64688) Putative pyruvate dehydrogenase complex E1 beta subunit
17.37	(-) (Q9LU41) Potential calcium-transporting ATPase 9, plasma membrane

Table 15

1 hr hypothetical genes

Fold Change	Genbank Description
5.316	(-) (Q9SVN9) Hypothetical 50.6 kDa protein
2.019	(+) (O23488) Hypothetical protein
2.371	(+) (Q94CK1) Hypothetical protein
3.58	(+) hypothetical protein
9.473	(-) MGC25461 hypothetical protein MGC25461
2.338	(-) hypothetical protein
3.218	(-) Hypothetical protein Rv3399/MT3507
2.549	(+) (Q9SUR3) Hypothetical protein (AT4G23630/F9D16_100)
2.001	(-) (CAD88219) Hypothetical protein
4.2	(-) PR-1 like protein

Table 16

2 hr hypothetical genes

Fold Change	Genbank Description
4.589	(+) hypothetical protein VSP-3
3.67	(-) (Q9STM2) Hypothetical
5.343	(-) (Q9LPM3) F2J10.10 protein (Hypothetical 23.3 kDa protein)
26.2	(+) Tax_Id=9606 Hypothetical protein KIAA1223
11.22	(-) hypothetical protein
3.51	(+) (Q9LXQ2) Hypothetical protein (At3g44100)
25.65	(-) hypothetical protein VSP-3
13.64	(-) (Q9LFN0) Hypothetical
2.363	(+) (Q9M343) Hypothetical
39.32	(+) (Q9GZF7) Hypothetical
10.57	(-) MGC27575 hypothetical protein MGC27575
2.508	(+) MGC36596 hypothetical protein MGC36596
2.047	(+) (Q9SVL4) Hypothetical
5.364	(-) (O49490) Hypothetical
3.757	(-) (O23551) Hypothetical
2.168	(+) (O23514) Hypothetical
6.417	(+) hypothetical protein
6.648	(-) Tax_Id=9606 Hypothetical protein FLJ35107 [Homo sapiens], 57.8% id
9.427	(-) hypothetical protein [Nostoc sp. PCC 7120], 96.5% id
20.83	(-) hypothetical protein [Nostoc sp. PCC 7120], 81.5% id

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