Expression of the bacterial *ect*ABC genes in the chloroplast of *Chlamydomonas reinhardtii*

60 study points

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"Everyone can rise above their circumstances and achieve success if they are dedicated to and passionate about what they do.” — Nelson Mandela

Dedication

I dedicate this work:

• To my mother and father for their unlimited love, support and for always believing in me.

• To my son James Besa Junior for the joy you have brought in my life.

• And to my relatives, friends, and colleagues.

Acknowledgements

This master project was done at the department of Molecular Biology at the University of Oslo, under the supervision of Professor Uwe Klein.
I would specially like to thank and acknowledge Professor Uwe Klein for sharing with me his extensive knowledge and practical laboratory skills during the duration of this project. There were times when I would panic during some experiments and I would call upon him for help and he was always ready to help. He showed exemplary guidance, patience, knowledge, availability, friendly attitude and constant encouragement throughout the course of this thesis. I attribute the level of my thesis to his encouragement and support because without him this project would not have been easy to complete. I could not have wished for a better supervisor.
To my son James Besa Junior, I know I owe you a "father-son bonding time," I will surely make up for the lost time now that I have come to the end of this project. I am looking forward to holding you in my arms and maybe teach you one or two important life lessons.
My sister Peggy, my sister in-law Regina and my brothers Mulenga and Kabanda, thank you for the encouragement and constant calls. Keep up with the same spirit.
I also would also want to pay special tribute to my parents for the undivided support and for positively guiding me through life.

Summary

Ectoine is a small compound that confers salt tolerance to several species of bacteria. Ectoine is produced after the expression of three genes, *ect*A, *ect*B and *ect*C.

There has been a marked increase in demand for ectoine based products in the medical, cosmetics and biotechnological industry in recent years, a demand which is not likely to abate anytime soon, suggesting the need for more ectoine based research.

The goal of this project was to express and determine whether or not codon optimized *ect*A, *ect*B and *ect*C genes in the chloroplast of the model organism *Chlamydomonas reinhardtii* would increase the cell`s salt tolerance.

The genes (*ect*A, *ect*B and *ect*C) were introduced into the chloroplast of *Chlamydomonas* by microprojectile bombardment, a method by which exogenous DNA is introduced into cells via high-velocity microprojectiles. Positive transformants were identified using a photosynthesis selection marker.

PCR (polymerase chain reaction) and gel electrophoresis were used for further analysis of the transformants. We had 50 transformants after photosynthesis selection, and out of these, we analyzed 11 transformants. Of the 11 transformants only one transformant (transformant 21) had all the three genes (*ect*A, *ect*B and *ect*C), and we chose to work with this transformant for the rest of the study.

Transformant 21 showed increased tolerance to salt. However, there is need to work on more samples in order to come to a firm conclusion.

ABBREVIATIONS

atpB	ATP synthase subunit beta
bp	Base pair
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
Kb	Kilobase pair
L	Litre
M	Molar
Mb	Megabase pair
mg	Milligram
mL	Milliliter
mRNA	Messenger RNA
ng	Nanogram
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
UV-light	Ultraviolet light
µg	Microgram
µL	Microliter

Contents

1. Introduction

1.1. Salt tolerant organisms and their hypersaline environments

Stress induced by salinity is one of the factors that affect growth and productivity in living organisms in all the three domains of life, archaea, bacteria and eukarya. Salinity has had contributed to the destruction of natural vegetation in many areas. This as a result has led to major changes to the landscape and the ecosystem.

High concentration of salt creates an osmotic effect that leads to cells losing water to the outside medium, resulting in their death. As a result, it has become prudent to develop species that can thrive in high saline environments and to understand the mechanisms salt tolerant organisms employ in order to produce bioactive osmotic compatible solutes which are of great significance for them to thrive in hostile high saline environments.

The increase in the concentration of salt in soils have had an adverse effect on agriculture from time immemorial. Current estimates are that about 20% of the world`s irrigated land is affected by salinity. This has increased the need for the discovery and eventual use of novel genes which could be incorporated into target organisms to enable them to thrive in high saline environments. The recent advances in biotechnology have made this possible. Hence it has become imperative to explore and try out a number of organisms including bacteria with beneficial genes in order to boost agriculture production (Das, 2015).

1.2. Halophilic and halotolerant bacteria

Bacteria able to survive in saline environments are grouped into halophilic and halotolerant bacteria respectively (Das, 2015).

Halophilic organisms (**Figure 1**) grow best in environments with high salt concentrations. They may be further classified into slight, moderate and extreme halophiles depending on their degree of salt requirement. Slight halophiles thrive well at 0.2–0.85 M (1–5%) sodium chloride (NaCl), while moderate halophiles thrive well at 0.85–3.4 M (5–20%) NaCl. For extreme halophiles, they grow optimally at 3.4–5.1 M (20–30%) NaCl concentration (DasSarma, 2012).

Halotolerant organism, in contrast, despite supporting a certain level of salt in their environment tend to thrive in environments which are devoid of salt. A microorganism is categorized as being extremely halotolerant, if it can grow in an environment having more than 2.5 M salt (Antón, 2011).

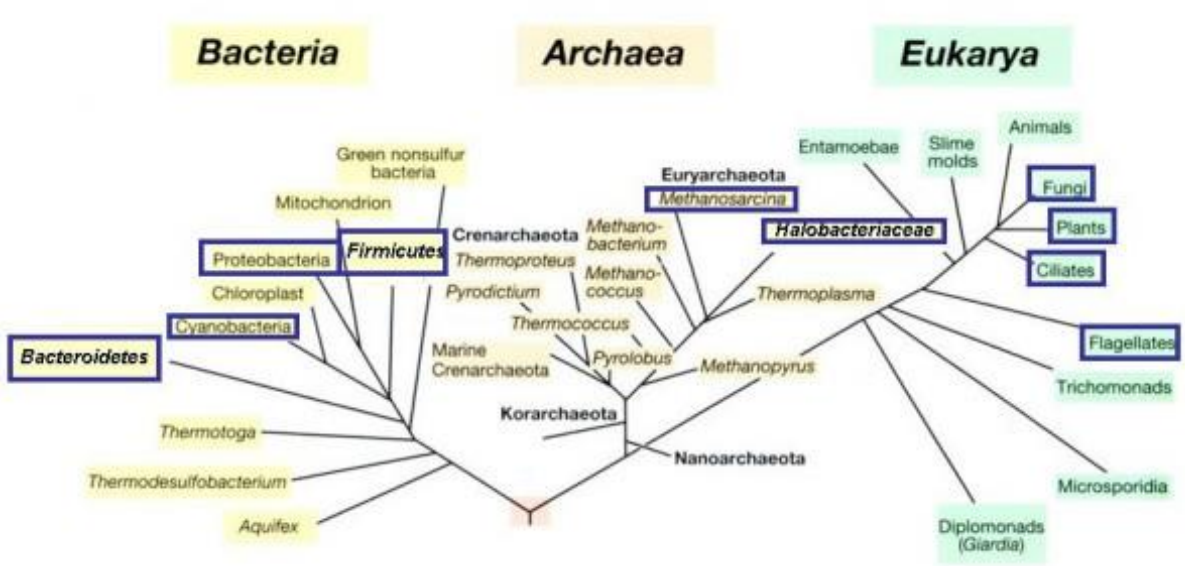


Figure 1. The distribution of organisms within the universal phylogenetic tree of life based on small subunit rRNA gene sequences. Groups marked with blue boxes contain at least one halophilic representative *(Oren, 2008)*.

1.3. Survival strategies

It is very cardinal for halophilic and halotolerant microorganisms to balance their cytoplasm with the surrounding medium, failure to which could lead to massive loss of water through plasmolysis. In order to prevent loss of cellular water, halophiles have developed a system whereby they accumulate high concentrations of solutes within the cytoplasm. The two main strategies microorganisms have developed in order to maintain an osmotic balance with the outside medium, and as a result enabling them to maintain their cell volume are called “salt-in” and “salt-out” (Oren, 2008).

1.3.1. Salt-in strategy

In this strategy cells are able to maintain their shape through the help of ion transporters. The ion transporters play a role in the influx of potassium (K^+) and chlorine ions (Cl^-) from the environment. In this strategy, the microorganism's enzymatic machinery is adapted to working in the presence of salt, hence there is need for the whole proteome to maintain its shape and activity at near-saturating salt concentrations. Organisms with this kind of system have acidic proteomes. When subjected to mediums with a low salt concentration, the proteins of these organisms denature. As a result, most of the microorganisms employing this kind of strategy fail to survive in low salt media. Generally, this strategy is widespread in extreme halophile microorganisms (Oren, 2008). **Figure 2,** shows some of the microorganisms using this kind of strategy.

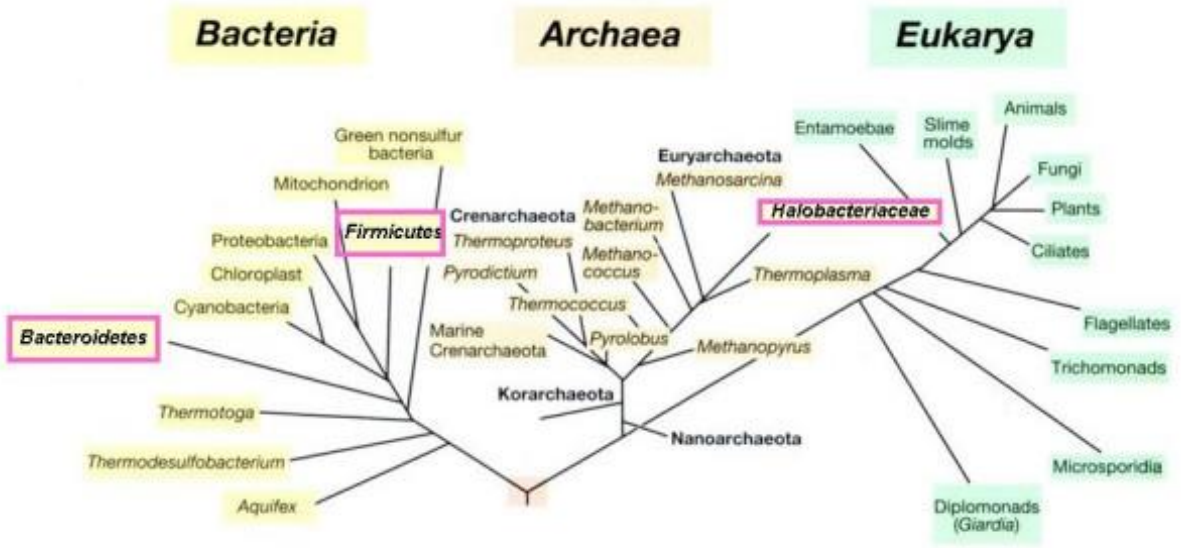


Figure 2. Distribution of organisms within the phylogenetic tree using the salt-in strategy. Groups marked with purple boxes contain microorganisms that use the salt-in strategy *(Oren, 2008)*.

1.3.2 Salt-out strategy

This strategy is widespread in nature. It is used by the majority of halophilic bacteria to maintain turgor. This one involves the biosynthesis and eventual accumulation of compatible solutes. Microorganisms employing this strategy exclude salt from the cytosol in preference for compatible solutes. These solutes in spite of their high concentration have no effect on the host cell`s enzymatic normal activity, as a result little to no adaption of the cells proteome is needed. In most cases organisms that employ this strategy can adapt to environments with a broad salt concentration range. This flexibility has been made possible due to the swift adjustment in the concentration of the compatible solutes in response to cues from the extracellular environment (Oren, 2008), (Das, 2015). **Figure 3** gives an overview of organisms using this type of strategy.

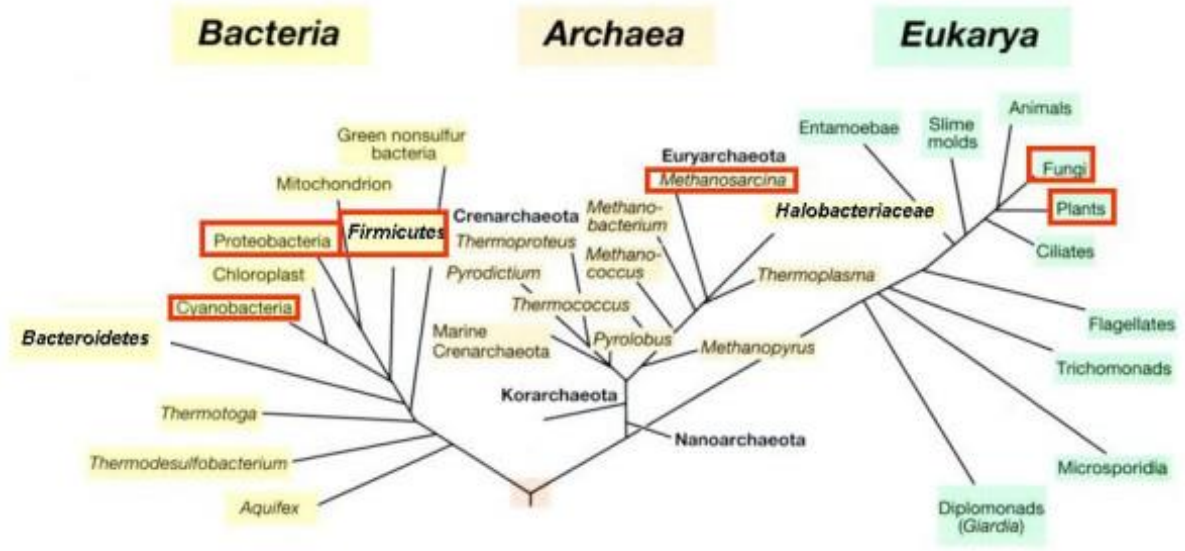


Figure 3. Distribution of organisms using the salt out strategy within the phylogenetic tree. Groups marked with red boxes contain at least some halophilic representatives in which de novo synthesis and/or accumulation of organic solutes have been demonstrated. From Oren (2008).

1.4. Compatible solutes

Compatible solutes are small osmotically active molecules. They are highly water soluble with a very low molecular weight. These molecules don't interfere with the cellular pathways in the host organisms, even though at times they interfered with some of the dynamic property of enzymes and other proteins. They are natural substances like alcohol, sugars, amino acids and their derivatives. Their main role is to help maintain an osmotic balance in the host cells, as a result helping to maintain protein stability. They are either made by the cell itself or are transported inside the cells from the environment. They play a role in helping cells to survive stressful environmental conditions like freezing, high temperatures and salt stress. Apart from helping cells to maintain their volumes, they also help in balancing of electrolytes (Das, 2015), (Bursy, 2008).

1.5. Ectoine

Ectoine is the most abundant compatible solute, it is found in several species of bacteria. It was first discovered in *Halorhodospira halochloris*, an extremely halophilic phototropic bacterium (Galinski, 1985), (Kolp, 2008). It has now been found in a number of gram- negative and gram-positive bacteria. Ectoine is a cyclic tetrahydropyrimidine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid;) compound with the molecular formula $C_6H_{10}N_2O_2$ (Galinski, 1985), (Kolp, 2008). The structure of Ectoine is shown in **Figure 4**.

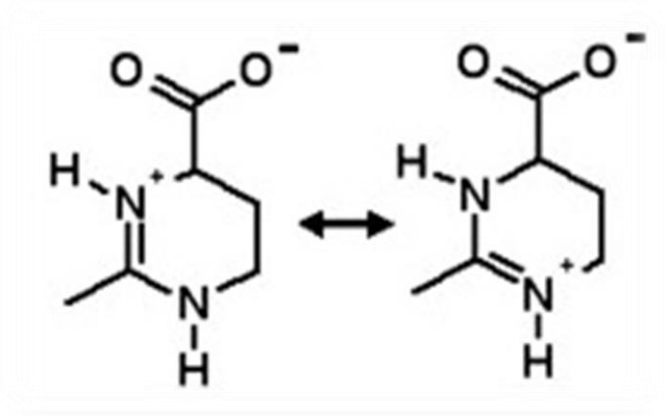


Figure 4. Molecular structure of ectoine showing its two tautomeric forms. From *(Graf, 2008)*.

1.5.1. Synthesis of ectoine

The proteins needed for the biosynthesis of ectoine are encoded by three genes, namely *ect*A, *ectB* and *ect*C. In some bacteria there is an additional gene called, *ect*D which codes for an enzyme, which further hydroxylates ectoine to 5-hydroxyectoine, which functions as a compatible solute in some bacteria.

The three genes (*ect*A, *ectB* and *ect*C) are organized into a single operon and are transcribed from a single promoter. These genes encode, L- 2,4-diaminobutyric acid acetyltransferase, L-2,4-diaminobutyric acid transaminase and L-ectoine synthase respectively.

The biosynthesis of ectoine begins with the conversion of aspartate-β-semialdehyde into L-2,4-diaminobutyrate by L-2,4-diaminobutyric acid transaminase (*ect*B). L-2,4-diaminobutyric acid is then further acetylated by L-2,4-diaminobutyric acid acetyltransferase (*ect*A) into Nγ-acetyl-L-2,4-diaminobutyric acid. After this stage, NaCl activates ectoine synthase (*ect*C), which subsequently converts Nγ-acetyl-L-2,4-diaminobutyric acid to ectoine (Rodríguez-Moyaa, 2012), (Bursy, 2008).

The biosynthetic pathway showing the conversion of L-aspartate-β-semialdehyde to ectoine/hydroxyectoine is shown in **Figure 5.**

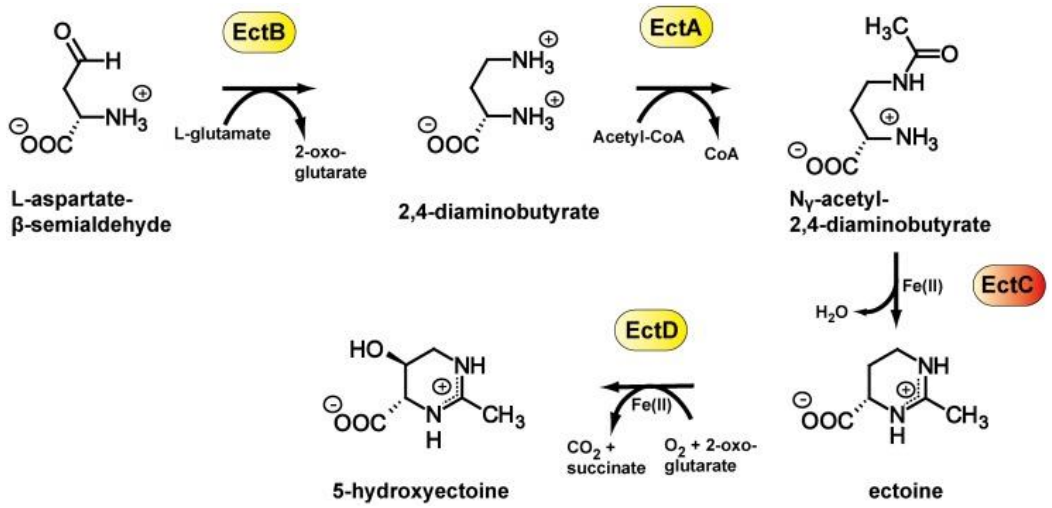


Figure 5. The biosynthetic pathway of ectoine and hydroxyectoine *(Widderich, 2016)*

1.5.2. Mechanisms used by ectoine in order to perform its osmoprotectant role

Two models have been proposed; the preferential exclusion model and the water replacement hypothesis.

The preferential exclusion model (**Figure 6**) states that compatible solutes are excluded from having any direct contact with the surface of the proteins, this helps the proteins to fold properly as well as interact with other proteins favourably. This process has a stabilizing effect on the native conformation and hence making denaturation thermodynamically less favorable. A layer of water forms in the space between the compatible solutes and the proteins. Consequently, the proteins occupy a smaller volume. Furthermore, the fact that the compatible solutes don't react with the proteins, helps to preserve their catalytic activity. The water hating hydrophobic parts of the proteins come together. These hydrophobic interactions are important for proper folding of proteins and their ability to remain in their native conformation (Pastor, 2010)

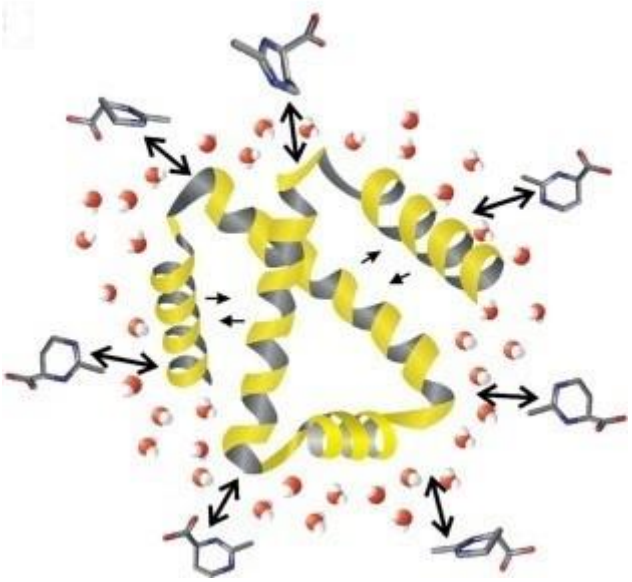


Figure 6. Protein stabilization mechanism of compatible solutes. Based on the preferential exclusion model. Small spheres represent water molecules and backbones represent compatible solutes (ectoine). From Pastor et al. (2010).

In contrast, the water replacement hypothesis is the complete opposite of the preferential interaction theory, due to the fact that in this theory water is replaced by solutes. The hypothesis holds that many organisms are able to lose over 50% of cellular water and still return to full activity when they get rehydrated. The proponents of this hypothesis believe that cellular structures are protected from degradation by the accumulation of certain compatible solutes. This hypothesis appears to be very important in extreme cases of low water activities, while the interaction model is the preferred model for the more diluted range of solute concentrations (Pastor, 2010).

1.5.3. Application of ectoine

Ectoine has raised a lot of interest in the biotechnology industry. It has been discovered that ectoine has an ability to help keep the skin moisturized, this has seen it being increasingly used an active ingredient in the cosmetic industry. As an effective osmolyte, ectoine has been found to help reduce stress which leads to aged skin. Furthermore, ectoine has found wide use in the sunscreen creams industry because of its ability to protect the skin from the sun`s UVA rays.

The fact that ectoine helps to protect the integrity of proteins as well as helping them to remain folded has led some scientists to call for more research on it, especially in the pharmaceutical industry where it has been suggested as a probable cure for diseases caused by misfolded proteins.

There is also potential for ectoine to become the answer to global food shortages through improved crop productivity and quality especially in areas where crops are failing because of high salinity in the soils. Undoubtedly there is need for more research on ectoine (Pastor, 2010), (Kolp, 2008).

1.5.4. Industrial production of ectoine

When it comes to the industrial production of ectoine, two key technologies are employed, namely, the bacterial milking procedure and the “Leaky” mutant procedure.

In the milking procedure, halophilic bacteria such as *Himanthalia elongata* are grown in a high saline medium. The bacteria are then suddenly subjected to a medium with a low salinity. This sudden change in salinity results in the cell to cope with a cytoplasm with an elevated osmolarity than the surrounding environment. This inevitably leads to an influx of water into the cell through the permeable cell membrane. The cell in an attempt to avoid bursting releases ectoine to the outside environment.

The excreted ectoine is collected and purified using cation exchange chromatography and crystallization processes. After the cells are harvested, the filtered cell mass is collected and put into high saline culture medium for another round of ectoine synthesis. The bacterial milking could be repeated within 10 hours.

Himanthalia elongata is preferred because of its fast regeneration time and its refined inherent ability to release ectoine in response to osmotic down shock (dilution stress).

In the case of the "Leaky" mutant procedure, a mutant which doesn't efficiently retain the compatible solutes inside the cell is used. Some of the bacteria have an ability to take up compatible solutes or the precursors for compatible solutes from the environment through their specific transporters and channels. However, because these cells are unable to retain the accumulated ectoine, it leaks out into the outside environment (medium). The leaked ectoine is then harvested from the medium (Kunte, 2014).

1.6. *Pseudomonas stutzeri* and the *ect*ABC gene cluster

Pseudomonas stutzeri is a gram-negative bacterium that synthesizes ectoine. It is found in almost all environments, which shows its adaptability. This bacterium has the *ect*ABC gene cluster organized as an operon (Stöveken et al., 2011), (Seip, 2011)

1.7. *Chlamydomonas reinhardtii* as a model organism

Chlamydomonas is a genus of unicellular photosynthetic green alga that was isolated from soil in North America. It has proved to be a model organism for the investigation of chloroplast photosynthesis, eukaryotic flagellum, cell-cell recognition and the processing of light signals. It has a simple genome, which has been fully sequenced.

Morphologically it is oval shaped. It is approximately 10 µm in diameter with two anterior flagella of 10 to 12 µm in length with which it swims with a breast-stroke type motion. The chloroplast makes up two thirds of its cytoplasm, while the rest of the cytosol is occupied by the nucleus, golgi apparatus endoplasmic reticulum, contractile vacuoles, mitochondria and other structures. It`s cell wall is composed of seven layers of glycoproteins. See **Figure 7** below.

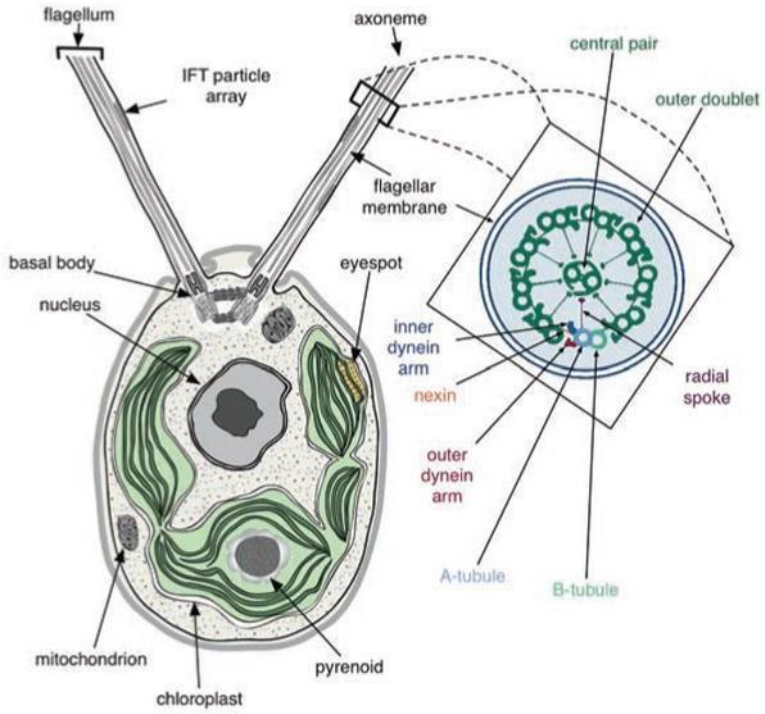


Figure 7. A schematic of a *Chlamydomonas* cell. Shown in the figure are the cell`s various organelles. Included is an expanded cross section of the flagellar axoneme, showing the nine outer doublets and the central pair microtubules. From Merchant et al. (2008).

Chlamydomonas has proved to be a very good candidate for research because of its inherent ability to grow heterotrophically in the absence of light, provided acetate is available in the medium, a feature which land plants lack. It has a fast generation time of approximately 6-8 hours and has an ability to grow in liquid or agar media. This makes it a very good candidate for scientific research.

Chlamydomonas` optimal growth temperature range is from 20 ° to 36 ° Celsius. The growth is faster if it is subjected to continuous light and extra carbon dioxide bubbling.

It has a linear mitochondrial genome of 15.8 kb; while the circular chloroplast genome is 203.4 kb in size, and lastly, its 17 linear nuclear chromosomes were found to be 121 Mb (Merchant, 2007), (Vahrenholz, 1993), (Maul, 2002).

1.8. Transformation

During the past decades, the demand for laboratory expressed proteins through recombinant means has increased. This demand is expected to markedly increase in the near future, hence there is need for more investment in this field. Currently the cost of production for most laboratory expressed proteins limits their availability. The undeniable demand for low-cost and high-yield laboratory expressed proteins is what is driving most biotechnology companies to research for faster, safer and more reliable protein expression systems.

In recent times, plant-based systems have proved to be a low-cost alternative in the production of these proteins. Initially, the expression of proteins in plants was based on integration of a target gene into the plant`s nuclear genome. It took time before a similar transformation was done in the chloroplast genome.

Plants produce proteins with posttranslational modifications akin to mammalian modifications. The production of proteins in plants eliminates chances of product contamination by mammalian pathogens. However, despite this, the amount of proteins expressed in nuclear transgenic plants is often low. There is also an increased chance of gene silencing, which can further contribute to reduced yields. Other reasons which have been cited for contributing to poor nuclear transformation includes, position effects due to random gene integration, chromatin structure and epigenetic effects.

Chloroplast transformation has proved to be a valuable alternative to nuclear transformation. Unlike nuclear transformation, chloroplast transformation has high protein expression levels. Chloroplast transformation eliminates chances of gene silencing and position effect frequently experienced in nuclear transgenic lines (Adem, 2017).

1.8.1. *Chlamydomonas reinhardtii* chloroplast transformation

The first stable transformation of chloroplasts was accomplished in 1989. This was when Boynton and co-workers managed to restore the photosynthetic capacity of a *C. reinhardtii* mutant through cell bombardment. They used high-velocity microprojectiles coated with the wild-type gene to achieve this feat. The chloroplast can contain in upwards of a hundred copies of the plastome. The majority of the genes in the plastomes have roles in the photosynthetic pathway, hence they are highly expressed.

Chlamydomonas reinhardtii has become a prime organism for study of photosynthesis, because of its efficient homologous chloroplast recombination system, making it easy to insert target genes into its plastome. This enables scientists to do precise and predictable site-specific expression. Consequently, many of the elucidated mechanistic aspect of excitation energy and electron transfer within oxygenic eukaryotic photosynthetic complexes were made possible using the *Chlamydomonas reinhardtii* model system. This has made it a protein expression system of choice (Mayfield, 2007), (Merchant, 2007).

1.8.2. Transformation by microprojectile bombardment

Microprojectile transformation has proved to be a very effective method for transferring exogenous DNA into cells. In this method, a gene gun or a biolistic particle delivery system is used to effectively deliver the DNA into the target cells. This is performed by using is a heavy metal coated with DNA. Tungsten or gold particles are used. The DNA coated tungsten or gold particles are shot onto the target cells. The high kinetic energy of the projectile (DNA coated tungsten or gold particles) enables the DNA not only to penetrate into the target`s cell wall, but also the double membrane of chloroplasts.

Once inside the chloroplast the exogenous DNA could be integrated into the chloroplast genome through homologous recombination, resulting in transformed *Chlamydomonas reinhardtii* (Sanford, 2007), (Klein & Sanford, 1987).

1.8.3. How genes are expressed in *Chlamydomonas reinhardtii*

The chloroplast of *Chlamydomonas reinhardtii* is widely used to express foreign proteins. The plastids genes of *Chlamydomonas reinhardtii* have their own independent protein- synthesizing machinery. Furthermore, the easiness and low cost involved in growing this alga as well as the high yield of recombinant proteins makes it an ideal foreign protein expression system (Mayfield, 2007).

1.9. Codon optimization

Codon optimization is a technique used to improve the expression of proteins in organisms by increasing the translational efficiency of target genes.

Amino acids are encoded by groups of three nucleotides, called codons. However, a single amino acid could be encoded by two or more different codons, or alternative nucleic acid sequences. Codons that encode the same amino acid are called synonyms. For example, CAU and CAC both encode histidine, hence are synonyms.

Not all transfer RNAs (tRNAs) are expressed equally across species, which can affect the protein expression levels, as a result there is need to optimize particular DNA sequence by changing their codons to match the most abundant tRNAs. For instance, if the most abundant tRNA recognizes the GUA codon, and the DNA sequence has CAC, the CAC could be changed to CAT, in the process increasing its translation efficiency.

Therefore, codon optimization is a necessity if increased protein production is the desired outcome (Potvin, 2010).

1.10. Ectoine production in *Chlamydomonas reinhardtii*

The *ect*ABC genes responsible for ectoine production have successfully been introduced into *Escherichia coli* bacteria resulting in a low level of ectoine production (Ninga, 2016). Similarly, this gene cluster was successfully introduced into tomato plants and the transgenic plants showed that the concentration of ectoine increased with increasing salinity compared to the wild-types (Moghaieb, 2011).

The *ect*A, *ect*B, *ect*C genes have been introduced and expressed in *Chlamydomonas reinhardtii* before without any noticeable increase in salt tolerance according to the findings of Anders Lunde, Nafisa Batool Afzal and Victoria Teigland Holck (Lunde, 2012), (Afzal, 2014), (Holck, 2014). This is probably due to the fact that they didn't optimize all the three genes before expressing them, which we did in this project.

2. Aim of project

The main goals were:

1. To express codon optimized *ect*A, *ect*B and *ect*C genes in *Chlamydomonas reinhardtii*.
2. To see if salt tolerance in *Chlamydomonas reinhardtii* increased after the integration of the optimized *ect*A, *ect*B and *ect*C genes.

The main goals were further subdivided into the following sub-goals

1. Ligate the optimized *ect*B gene into a construct containing *ect*A/C gene sequences.
2. Clone the optimized *ect*A, *ect*B and *ect*C genes into the chloroplast expression vector.
3. Insert the *ect*A, *ect*B and *ect*C genes into the chloroplast of *Chlamydomonas reinhardtii* by microprojectile bombardment.
4. Detect *ect*A, *ect*B and *ect*C gene expression through reverse transcription polymerase chain reaction
5. Determine if there is increased salt tolerance among the selected transformants.

3. METHODS AND MATERIALS

3.1. Work with Escherichia coli

3.1.1. Preparation of agar growth plates with ampicillin

Procedure for 1 L (approximately 40 plates):

-1 L of lysogeny broth (LB) with 1.5% agar (check appendix 4) is prepared and autoclaved.

-The solution is cooled down to about 50ºC (Ampicillin is sensitive to temperature degradation), and 1 mL of ampicillin (60 mg/mL) is added, to a final concentration of 60 µg/mL.

-The solution is poured into sterile petri dishes under a sterile hood, and allowed to solidify.

-After solidification, the dishes are turned upside down (to avoid water condensation) and stacked. They are stored in sealed plastic bags at 4ºC.

3.1.2. Transformation of competent *E. coli* cells

Procedure:

-Melt an aliquot of frozen competent cells on ice, and add 3µL pure DNA (1 ng/µL) or 3 µL of a ligation reaction. Mix and leave tube on ice for 30 minutes.

-Incubate at 42 ºC for 90 seconds. Immediately cool down on ice 1-2 min.

-Add 0.8 mL sterile LB medium at room temperature to a 15 mL plastic tube, and transfer the cell mix to the tube. Grow the cells for 1 hour on a rotating wheel at 37ºC

-Plate the suspension (see next page).

3.1.3. Application and growth of *E. coli* on agar plates

*This procedure is done in a sterile hood.

Procedure:

-A glass rod is sterilized by burning with alcohol and cooled down in air.

-75 µL of *E. coli* culture is pipetted onto agar plate (dilute the culture if too many colonies appear or spread out on more dishes).

-The petri dish is put on a hand operated turntable and rotated while the glass rod is used for spreading the *E. coli* culture evenly around the plate.

-The plate is left for a few minutes to allow the liquid to be absorbed into the agar.

-The lid is put on, and then the petri dish is turned upside-down, and incubated at 37 ºC for 16-20 hours.

-Store at 4ºC or use immediately. (Can be stored in the cold for several weeks).

3.1.4. Culturing of *E. coli* agar plate colonies for minipreparations

Procedure:

-3 mL of lysogeny broth (LB) with ampicillin (60 µg/mL) is added to a sterile 15 mL tube with loose cap for air ventilation.

-A single colony is picked from a plate, with a sterile pipette tip.

-The pipette tip is put inside the tube, making sure the tip with the colony is in contact with the LB medium.

-The tube is incubated overnight at 37ºC on a rotating wheel.

-The next day, the culture can be used for plasmid isolation (Miniprep).

3.1.5. Storage of *E. coli* at -80ºC

Procedure

-Take 0.7 mL of the *E. coli* culture and mix with 0.3 mL of 50% glycerol in a 2 mL tube with screw cap.

-Store in -80ºC freezer.

3.1.6. Miniprep: plasmid isolation from *E. coli*

Procedure:

-Transfer 1.5 mL of an *E. coli* culture into a 1.5 mL tube. Store the rest of the culture in a cold room or refrigerator (4ºC).

-Centrifuge at 13 000 rpm in room temperature for about 30 seconds.

-Discard supernatant, leaving the bacterial pellet as dry as possible.

-Resuspend the bacterial pellet by vortexing in 100 µL ice cold TEG buffer.

-Leave the mixture for 5 min at room temperature.

-Add 200 µL of NaOH/SDS (0.2 N, 1% (w/w)) solution (prepare fresh). Mix by inversion, and incubate on ice for 5 min.

-Centrifuge at 13 000 rpm at 4ºC for 5 min.

-Transfer the supernatant to a new microfuge tube, and add 410 µL of phenol/chloroform/isoamylalcohol (25:24:1). Mix by vortexing.

-Centrifuge at 13 000 rpm at room temperature for 2 min.

-Transfer upper phase to a new microfuge tube. Add 410 µL of chloroform/isoamylalcohol (24:1). Mix by vortexing.

-Centrifuge at 13 000 rpm at room temperature for 2 min.

-Transfer 310 µL of upper phase to a new tube. Add 750 µL of ice-cold 96% ethanol. Mix by vortexing and leave on ice for 10 min to precipitate nucleic acids.

-Centrifuge at 13 000 rpm at 4ºC for 10 min.

-Discard supernatant, and add 1 mL of ethanol (70%) to the pellet. Mix by inversion.

-Centrifuge at 13 000 rpm at 4 ^{o}C for 5 min.

-Discard supernatant, and dry the pellet by leaving at room temperature for about 10 minutes, or dry in a vacuum centrifuge.

-Dissolve the pellet in 15 µL of sterile distilled water.

- Use immediately, or store in freezer at 20^{o}C.

*A yield of 1 to 3 µg of plasmid DNA can be expected (for more scale up the procedure). The preparation contains RNA, which will be removed with ribonuclease A when the DNA is digested with restriction enzymes.

3.1.7. Maxiprep: plasmid isolation from *E. coli*

Procedure:

-Inoculate 5 µL or 1 colony from a plate of *E. coli* culture in 100 mL LB with ampicillin (60 µg/mL), and grow the culture overnight at 37^{o}C on a shaker.

-Centrifuge at 6000 rpm at 4^{o}C for 5 min.

-Discard supernatant, and resuspend cells in 3.6 mL ice-cold TEG buffer.

-Add 0.4 mL of lysozyme (10 mg/ml) in TEG (prepare fresh). Leave at room temperature for 5 minutes, and then leave on ice for 5 minutes.

-Add 8 mL NaOH/SDS (0.2 N, 1% (w/w)) solution (prepare fresh). Mix by inversion and leave on ice for 5 minutes.

-Add 6 mL ice-cold potassium acetate (5M K, 3M acetate). Mix by inversion, and incubate on ice for 5 min.

-Centrifuge for 10 minutes at 6000 rpm and at 4^{o}C.

-Transfer supernatant to a new tube by filtering through a gauze. Add 12.5 mL isopropanol. Mix by vortexing. Leave at room temperature for 15 min.

-Centrifuge for 10 minutes at 6000 rpm in room temperature.

-Discard supernatant, and leave tube to dry in room temperature for 10 minutes.

-Resuspend pellet in 3mL TE (50mM Tris (pH 8), 1mM EDTA) buffer.

-Determine the weight of the solution and add TE (50mM Tris (pH 8), 1mM EDTA) buffer to 4.2 grams.

-Add 4.5 g CsCl, warm solution with your hands to reach room temperature while mixing. Make sure all the CsCl dissolves.

-Add 0.25 mL ethidium bromide (10 mg/ml) and 0.25 ethidium bromide (10 ng/mL). Mix by pipetting.

-Centrifuge for 5 minutes at 6000 rpm in room temperature.

-Transfer supernatant to Beckman OptiSeal tubes, and load tube into VTi 65.2 rotor.

-Centrifuge at 50 000 rpm for minimum 15 hours at 15^{o}C. Decelerate rotor without brake.

-Carefully transfer the ultracentrifugation tube to a clamp, and illuminate the tube with 350 nm UV-light.

-Extract the middle band with a 2 mL syringe. The volume should be approximately 0.5 mL.

-Remove ethidium bromide by extracting with 0.75 mL isopropanol/water (7:1 v/v) up to five times.

-Dialyze against sterile 200 mL TE buffer at 4^{o}C for about 2 hours. Replace with fresh buffer once every 20 minutes.

-Transfer the DNA solution to a tube with screw cap.

-Determine the concentration by measuring the absorption at 260 nm (dilution 1:100).

-Store in the freezer at 20^{o}C.

3.2. Work with DNA

3.2.1. Quantification of DNA by Ultra violet light absorption

Procedure:

-Calibrate/zero the spectrophotometer at 260 nm with a control sample, using the liquid used to resuspend the DNA.

-Dilute sample as needed, and measure optical density (OD).

-OD 1.0 = 50 µg DNA/mL (40 µg RNA/mL).

3.2.2. Quantification of DNA by "Dot spot"

Procedure:

-Prepare a standard of DNA in water solutions, with increasing concentrations (0 ng/µL, 2.5 ng/µL, 5 ng/µL, 7.5 ng/µL, 10 ng/µL and 15 ng/µL).

-Place a series of 2 µL drops of ethidium bromide-water solution (2 µg/mL) on a plastic petri dish that is transparent to UV-light. Place 6 drops for the DNA-standards, and add additional drops according to the number of samples you wish to measure.

-Add 2 µL of the DNA-standards to each ethidium bromide-water drop, mixing by pipetting within the drop a few times.

-Add 2 µL of the sample DNA to the rest of the ethidium bromide-water drops, mix by pipetting. Usually up to a 100-fold dilution is required to lower the sample DNA-concentration to match that of the standards range.

-Compare the sample fluorescence and the standards with UV-light, and estimate the DNA concentration.

3.2.3. Agarose gel electrophoresis

Procedure (1% agarose gel):

-Measure 60 mL TAE (1x) buffer in an Erlenmeyer flask.

-Add 0.6 g agarose.

-Use a microwave oven to make the mixture boil (you may use a plastic foil to cover the flask so you do not spill). Make sure all the agarose is dissolved.

-Cool down the Erlenmeyer flask to about 45 ^{o}C and add 10 µL of ethidium bromide (1 mg/mL).

-Pour the solution into a tray, and insert comb.

-Wait for solidification, and immerse the gel in an electrophoresis chamber. Pour TAE (1X) buffer into the chamber so that it just submerges the gel.

-Mix the DNA sample with 1-2 µL of loading buffer. Load X µL in each well (depending on which comb is used). In most cases: remember to load a control and a DNA ladder.

-Apply a voltage according to electrophoresis chamber specifications. Usually 90 volts.

-After ca. 45-60 minutes the electrophoresis is finished. Visualize the DNA by using UV-light.

3.2.4. Purification of DNA fragments from agarose gel

*Specific DNA samples were isolated and purified by using the IllustraTM purification kit from GE Healtcare, following the protocol that comes with the kit.

Procedure:

-Cut out an agarose slice which contains the DNA fragment of interest, and put it into a 1.5 mL Eppendorf tube.

-Calculate the weight of the agarose gel by weighing.

-Add 10 µL Capture buffer to each 10 mg agarose gel. Mix by inversion.

-Use a heating block at 60ºC to melt the agarose (ca. 10 minutes).

-Add 600 µL sample to a microspin column and collection tube. Leave at room temperature for 60 seconds.

-Centrifuge for 30 seconds at 13 000 rpm in room temperature. Discard flow through. Repeat this step until the whole sample is used.

-Add 500 µL wash buffer. Centrifuge for 60 seconds at 13 000 rpm in room temperature.

-Transfer spin column to a clean, DNase-free microcentrifuge column.

-Use 10-50 µL elution buffer, and leave at room temperature for 60 seconds.

-Centrifuge for 60 seconds at 13 000 rpm in room temperature.

-Store DNA sample (flow through) at -20ºC, or use immediately.

3.2.5. Precipitation of DNA

Procedure:

-Add Na-acetate so that it makes up 10% of the volume in the DNA solution.

-Add 96% ethanol so it makes up 2/3 of the total sample volume.

-Freeze at -20ºC for minimum 30 minutes (can also freeze overnight).

-Centrifuge at 4ºC for 10 minutes at 13 000 rpm.

-Remove all liquid (make sure you do not lose the pellet).

-Add 1.0 mL of 70% ethanol.

-Centrifuge at 4ºC for 5 minutes at 13 000 rpm.

-Remove all liquid and dry with the vacuum centrifuge for about 1-2 minutes.

3.2.6. Dephosphorylation of DNA

Procedure:

-Resuspend precipitated DNA in 90µL dH_2O and add 10 µL of buffer which came with the kit.

-Add 1µL Calf-intestinal alkaline phosphatase (CIP) enzyme, and mix with pipette.

-Put the sample on a heating block at 37ºC for 1 hour.

-Inactivate CIP by adding 100 µL of phenol/chloroform/isoamylalcohol (25:24:1). Mix by vortexing.

-Centrifuge at 13 000 rpm at room temperature for 2 min.

-Transfer upper phase to a new microfuge tube, and add ca. 100µL of chloroform/isoamylalcohol (24:1). Mix by vortexing.

-Centrifuge at 13000 rpm at room temperature for 2 min.

-Transfer the upper phase to a new tube. Add 200 µL of ice-cold 96% ethanol. Mix by vortexing and leave on ice for 10 min to precipitate nucleic acids.

-Centrifuge at 13000 rpm at 4ºC for 10 min.

-Discard supernatant, and add 1 mL of ethanol (70%) to the pellet. Mix by inversion.

-Centrifuge at 13000 rpm at 4ºC for 5 min.

-Discard supernatant, and dry the pellet by leaving at room temperature for about 10 minutes, or dry in a vacuum centrifuge.

-Dissolve the pellet in 15 µL of sterile distilled water.

3.3. Cloning

3.3.1. Ligation reaction

*T4 DNA ligase was used for all the ligation reactions. The amount of insert used was based on molar ratio 1.3:1 (insert: vector). The amount of vector used in a 10 µL reaction was 800 ng of a vector of 12 kb, and 400 ng of a vector of 6 kb and so on.

Procedure (10 µL):

-Calculate concentration and ratio of vector and insert. Total sample volume should be 6.5 µL. Use dH_2O if necessary.

-Leave the mixture on a heating block at 45^{o}C for 5 minutes.

-Cool down on ice for 1-2 minutes.

-Add 1 µL T4 DNA ligase buffer (10x), 2 µL PEG 8000 (30% w/v) and 0.5 µL T4 DNA ligase.

-Incubate at 19^{o}C for minimum 3 hours.

-Use immediately or store at -20^{o}C.

3.3.2. Digestion with restriction enzymes

Procedure:

All restriction digestions were done using enzymes from NEB (New England Biolabs). We followed the protocols as recommended by the supplier. The final restriction mixture was a mix of Enzyme, DNA, dH_2O, buffer and if needed BSA.

3.3.3. Plasmids

The PSB01 plasmid, carrying the *ect*ABC gene cluster from *Pseudomonas stutzeri* was provided by Dr. Galinski (University of Bonn, Germany). These genes needed to be optimized for optimum expression in *Chlamydomonas*, the process is shown in **3.3.4,** while how the transformation plasmids were constructed is shown in **3.4.**

3.3.4. Codon optimization of *ect*A, *ect*B and *ect*C

The codons of *ect*A, *ect*B and *ect*C were optimized using the online tool "Graphical codon usage analyser" (http://gcua.schoedl.de/sequential_v2.html). In addition, the function "each triplet position vs usage table" was used. This was done using the codon usage table at (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055.chloroplast). **Table 1** on the next page shows the Codon usage in the *Chlamydomonas reinhardtii chloroplast.*

Table 1. Codon usage in the *C. reinhardtii chloroplast* (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055.chloroplast)

chloroplast Chlamydomonas reinhardtii [gbpln]: 93 CDS's (26731 codons)

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 33.4(894)	UCU 17.0(455)	UAU 24.6(657)	UGU 7.6(203)
UUC 17.1(456)	UCC 2.8(74)	UAC 10.0(266)	UGC 1.5(39)
UUA 77.7(2078)	UCA 22.0(588)	UAA 2.9(78)	UGA 0.1(3)
UUG 4.3(114)	UCG 4.0(107)	UAG 0.4(12)	UGG 13.5(361)
CUU 14.3(383)	CCU 15.5(414)	CAU 10.1(270)	CGU 32.4(866)
CUC 1.0(28)	CCC 3.4(90)	CAC 8.8(235)	CGC 4.1(110)
CUA 6.4(170)	CCA 23.6(630)	CAA 38.4(1026)	CGA 3.4(90)
CUG 3.7(99)	CCG 2.4(63)	CAG 4.1(110)	CGG 0.5(14)
AUU 51.4(1374)	ACU 24.4(651)	AAU 42.1(1126)	AGU 16.0(428)
AUC 8.2(219)	ACC 5.1(135)	AAC 17.7(472)	AGC 5.4(144)
AUA 6.9(184)	ACA 32.4(865)	AAA 69.1(1847)	AGA 5.3(143)
AUG 22.3(596)	ACG 3.9(103)	AAG 6.2(167)	AGG 0.9(23)
GUU 29.3(783)	GCU 34.0(908)	GAU 25.3(676)	GGU 44.0(1177)
GUC 2.5(68)	GCC 5.9(159)	GAC 9.8(263)	GGC 6.4(172)
GUA 26.0(696)	GCA 20.7(554)	GAA 41.1(1098)	GGA 8.6(229)
GUG 5.6(149)	GCG 3.3(88)	GAG 5.7(152)	GGG 3.7(99)

Coding GC 33.72% 1st letter GC 44.40% 2nd letter GC 37.35% 3rd letter GC 19.40%

3.4. Constructing the transformation plasmid

After the codon optimization of the *ect*A, *ect*B and *ect*C genes was done, the gene constructs were synthesized by Life TechnologiesTM GeneArt® service.

The *ect*B construct was designed in such a way that the *ect*B gene was linked to the 5' region of the *Chlamydomonas* chloroplast psbD gene and the 3' region of the *rbcL* gene. This construct was delivered to us in the pMA-RQ plasmid.

3.5. Work with *Chlamydomonas reinhardtii*

3.5.1. *Chlamydomonas reinhardtii* strains

Both the non-photosynthetic atpB deletion mutant strain ac-uc-221 (CC373) and the wild type strain (CC125) of *Chlamydomonas reinhardtii* were originally obtained from the culture collection of the *Chlamydomonas* Genetics Centre at University of Minnesota, MN, USA.

The photosynthesis mutant is maintained in the dark, in high salt high acetate (HSHA) media, while the wild type and transformants are maintained in the high salt (HS) media. The mutants are unable to perform photosynthesis, and die when exposed to light in a medium lacking an organic carbon source.

3.5.2. Preparation of solid media for *Chlamydomonas reinhardtii*

Procedure (1 L ca. 40 plates):

-Prepare 1 L of high salt and high acetate/high salt (HSHA/HS) media with 1.5% agar. Autoclave for 20 minutes.

-The solution was cooled down to 50^{o}C, and poured into sterile petri dishes under a sterile hood. The solution needs time to solidify.

-After solidification the plates are stacked, turned upside down (to avoid water condensation), sealed in plastic bags, and stored at room temperature.

3.5.3. Preparation of liquid medium for *Chlamydomonas reinhardtii*

Procedure (1 L):

-Prepare 1 L of HSHA/HS media in one large Erlenmeyer flask, or 250 mL flasks.

-Autoclave for 20 minutes.

-Store at room temperature.

3.5.4. Culturing the *Chlamydomonas reinhardtii* photosynthesis mutants for transformation

Procedure:

-Inoculate a 100 mL solution of high salt high acetate (HSHA) medium with the photosynthesis mutant *Chlamydomonas reinhardtii*.

-Shield the culture from light by wrapping the flask in dark paper, and leave the culture on a shaker for 2 days.

-Inoculate ca. 5 mL of the culture to a new flask with 100 mL HSHA media, and leave the culture on the shaker for 2 days.

- Inoculate this culture to a new flask with 500 mL HSHA media, and leave the culture on a shaker for 1 day. Remember to always shield the culture from light.

-The culture will be ready for transformation.

3.5.5. Harvesting and plating of the *Chlamydomonas reinhardtii* photosynthesis mutant for transformation

Procedure (6 plates):

-Centrifuge 500 mL of the *Chlamydomonas reinhardtii* mutant culture in two sterile centrifuge tubes at 5000 rpm at room temperature for 5 minutes.

-Discard the supernatant and resuspend the cells in up to 500 µL of high salt high acetate (HSHA) media.

-Heat sterilize soft agar (0.11% agar in HSHA) in a microwave oven, and add 900 µL to two sterile microfuge tubes.

-Let the tubes cool down to 42^{o}C and add 100 µL of the resuspended *Chlamydomonas reinhardtii* mutant cells to each microfuge tube, and mix gently.

-Transfer 300 µL of the cells to an HSHA agar plate (3 cm in diameter). Spread the liquid as evenly as possible on the plates. Do the same for all 6 plates. Do not turn the plates upside-down.

-Store the plates in the dark for minimum 3 hours before microprojectile bombardment

*All steps should be done with as little light as possible.

3.5.6. Microprojectile bombardment

Procedure:

-Transformation vector DNA (5µg) is precipitated onto gold particles according to instructions of the manufacturer of the particle delivery system (Biorad).

-The following steps below are done in low light.

-The whole inside of the particle bombardment device is sterilized by wiping with ethyl alcohol.

-The particle bombardment device is assembled with the agar plates with photosynthesis mutant, and particle bombardment is carried out according to operating instructions.

-After bombardment, the plates are stored in the dark at room temperature for 1 day in order for the cells to recover. Do not turn upside-down.

3.5.7. Recovery and selection of transformants

Procedure:

-1 day after microprojectile bombardment, the bombarded plates are transferred to a sterile hood.

-400 µL of high salt (HS) media is added to each of the small plates.

-A hand held tool with a ridged metal wire angled to 90^{o}C is used to scrape off the thin layer of cells that are embedded in the soft agar. As many cells as possible are scraped into the HS medium that was added to the plates.

-All of the liquid on the plates is then transferred to a fresh HS agar plate with a pipette and spread out as evenly as possible using a glass rod. Do the same with all the plates.

-The HS agar plates are placed in the light. Do not turned upside-down, the liquid needs a day to dry.

-After 1 day, the plates are sealed with parafilm, and turn upside-down. They are kept under constant light.

-Colonies of transformed *Chlamydomonas reinhardtii* should appear after approximately 2 weeks. These are picked in a sterile hood and transferred to HS agar plates with a visual grid, and later inoculated in liquid cultures.

3.5.8. Photosynthetic growth of *Chlamydomonas reinhardtii*

When growing *Chlamydomonas reinhardtii* photosynthetically the algae are first cultured on solid media, and then later in liquid media, with no carbon-source other than CO_2 from the air. The algae are under constant light.

3.5.9. Photosynthetic growth of *Chlamydomonas reinhardtii* with 2% CO_2

*Prior to DNA and RNA isolation *Chlamydomonas reinhardtii* is grown with an additional supply of CO_2 in liquid culture, and in a 12 hours light/12 hours dark cycle.

Procedure:

-Add 100 mL of high salt medium (HS) to a 250 mL glass tube that has both gas inlet and outlet enabling bubbling by air, and the tube should otherwise be sealed in order to prevent contamination. This should be done in a sterile hood.

-Inoculate to a tube with about 200 mL of liquid culture.

-Place the tube in a water bath with a constant temperature of 30^{o}C.

-Connect the gas inlet to a source that supplies 2% CO_2 in to the air. This is done by bubbling CO_2 and air trough water, at a rate of e.g. 2 bubbles per second, leading the gases into the *Chlamydomonas reinhardtii* culture. The exact amount and rate of bubbling will (only) influence the speed of growth and can be varied.

-After a few days the cultures should be dark green and ready for harvesting.

3.5.10. Total DNA isolation from *Chlamydomonas reinhardtii*

Procedure:

-Centrifuge 40-80 mL (depending on how long the cells have grown, and hence their concentration) of a *Chlamydomonas reinhardtii* culture containing about 2 x 106 cells per mL for 5 minutes in room temperature at 5000 rpm.

-Discard supernatant and resuspend the pellet in 0.75 mL DNA extraction buffer (100 mM Tris pH 8.0, 50 mM Na2-EDTA, 0.5 M NaCl, 10 mM β-mercaptoethanol), and transfer the suspension to a 2 mL microfuge tube.

-Add 60 µL SDS (21 % w/v), mix, and incubate for 15 minutes at 65ºC. While at 65ºC, mix by inversion every 4 minutes.

-Let the mix cool down to room temperature, and add 0.9 mL phenol (equilibrated with 0.1 M Tris pH 8.0). Mix gently by inversion.

-Centrifuge for 5 minutes in room temperature at 13 000 rpm.

-Transfer 750 µL of the upper phase to a new 2 mL microfuge tube, and add 750 µL of phenol/chloroform/isoamylalcohol (25:24:1). Mix by inversion.

-Centrifuge for 5 minutes in room temperature at 13 000 rpm.

-Transfer 650 µL of the upper phase to a new 1.5 mL microfuge tube, and add 650 µL of isopropanol. Mix by inversion and incubate at room temperature for 5 minutes (or more, until precipitate appears).

-Centrifuge for 2 minutes in room temperature at 4000 rpm. Discard supernatant, and add 1 mL of ice-cold ethanol (70%). Mix by inversion.

-Centrifuge for 2 minutes in room temperature at 13 000 rpm. Discard supernatant, and dry pellet in a vacuum centrifuge (or by incubation at room temperature).

-Resuspend pellet in 90 µL TE buffer (10 mM tris pH 8.9, 1 mM Na2-EDTA), add 10 µL RNase A (1 mg/mL), and incubate at 37ºC for 1 hour.

-Extract the mixture once with 100 µL phenol/chloroform/isoamylalcohol (25:24:1), and once with 100 µL chloroform/isoamylalcohol (24:1).

-Precipitate the DNA in freezer for up to 1 hour with ethanol by adding Na-acetate so the volume is 10% of the total volume, and then adding 2 final volumes ethanol (96%).

-Centrifuge for 10 minutes in 4ºC at 13 000 rpm. Discard supernatant, and add 1 mL of ice-cold ethanol (70%). Mix by inversion.

-Centrifuge for 5 minutes in 4ºC at 13 000 rpm. Discard supernatant, and dry pellet in a vacuum centrifuge (or by incubation at room temperature).

-Resuspend DNA in 20 µL off sterile dH2O. Measure DNA concentration by dot spot (see section 3.2.2), and store in freezer (-20ºC) or use immediately.

3.5.11. RNA isolation from *Chlamydomonas reinhardtii*

*Samples most always be kept on ice and in the beginning, you should try to work as quick as possible. All the solutions and consumables are RNase free.

Procedure:

-Centrifuge 40 mL of a *Chlamydomonas reinhardtii* culture containing approximately 2 million cells per mL for 5 minutes at 5000 rpm in 4ºC.

-Discard the supernatant and resuspend the pellet in 1.5 mL ice-cold lysis buffer (0.6 M NaCl, 200 mM tris pH 8.0, 10 mM Na2-EDTA).

-Add 150 µL RNase inhibitor (200 mM vanadyl ribonucleoside (NEB)).

-Transfer 3 mL of the upper phase to another 15 mL tube containing 3 mL ice-cold phenol/chloroform/isoamylalcohole (25:24:1) and mix.

-Centrifuge for 5 minutes at 8000 rpm in 4ºC and transfer 2.5 mL of the upper phase to another 15 mL tube containing 2.5 mL ice-cold phenol/chloroform/isoamylalcohole (25:24:1) and mix.

-Centrifuge for 5 minutes at 8000 rpm in 4ºC, and transfer 2 mL of the upper phase to another 15 mL tube containing 2.5 mL ice-cold isopropanol and 250 µL Na-acetate (3 M pH 5.2). Mix and incubate at -20ºC for minimum 1 hour.

-Centrifuge for 15 minutes at 12 000 rpm in 4ºC. Discard supernatant, and leave the tube upside-down on a paper towel for 10 minutes in order to completely dry the pellet. Make sure that the pellet is not loose when you turn the tube upside-down, or the pellet might be lost.

-Resuspend pellet in 300 µL DEPC-treated H_2O, and transfer to a sterile 1.5 mL microfuge tube containing 100 µL ice-cold LiCl (8 M). Mix and incubate on ice for 2 hours.

-Centrifuge for 30 minutes at 13 000 rpm in 4ºC. Discard supernatant and resuspend pellet in 100 µL DEPC-treated H_2O, and keep on ice.

-Dilute 10 µL of the RNA solution in 1 mL DEPC-treated H_2O, and measure OD260 nm (OD 1.0 = 40 µg RNA/mL). To the rest of the DNA solution add 10 µL Na-acetate (3M pH5.2) and add 200 µL ice-cold ethanol (96 %). Mix and incubate at -20ºC for minimum 1 hour.

-Centrifuge for 10 minutes at 13 000 rpm in 4ºC. Discard supernatant and add 1 mL of ethanol (70 %), mix by inversion.

-Centrifuge for 10 minutes at 13 000 rpm in 4ºC. Discard supernatant and dry the pellet in a vacuum centrifuge.

-Resuspend RNA to a concentration of 2 µg/µL in DEPC-treated H_2O.

3.6. Analytical methods

3.6.1. DNA sequencing

In order to confirm the correct sequence orientation of the *ect*A, *ect*B and *ect*C genes, we sent our DNA samples to Eurofins in Germany for sequencing. Knowing the orientation of the *ect*A, *ect*B and *ect*C genes helped us to know the expected sizes of the fragments after enzyme digestion.

3.6.2. Polymerase Chain Reaction (PCR)

PCR was performed in a T1 Thermocycler (Biometra). The Taq polymerase and other reagents were supplied by New England BioLabs. The ectoine primers used were synthesized by Eurofins Genomics. The primer sequences are in section 3.6.2.2

Recipe for 25µl PCR mixture:

-In a PCR tube add 5µl (5X) buffer

-Add 0.75 µl dNTP

-DNA 5 µl template.

-Add 0.5µl DNA polymerase

-Add 1.25µl of forward primer and 1.25µl of reverse primer

-Add 11.25µl distilled water

PCR reaction

Step 1: 94°C, 3 minutes

Step 2: 94°C, 15 seconds

Step 3: 54°C, 1 minutes.

Step 4: 74°C, 45 seconds, repeat steps 2-4 30 times.

Step 5: 74°C, 10 minutes

3.6.2.1. cDNA synthesis

The enzyme reverse transcriptase uses an RNA template and a short primer complementary to the 3' end of the RNA to synthesise the first strand cDNA, which can further be used directly as a template for the Polymerase Chain Reaction (PCR).

Synthesis of cDNA suitable for PCR amplification was done using a RevertAid First Strand cDNA Synthesis Kit supplied by Fermentas.

Procedure:

- In a tube on ice mix 5µl total RNA, 1µl sequence-specific primer (20pmol).
- Add DEPC-treated water to the final volume 12µl.
- Mix gently and spin down for 3-5 seconds in a microcentrifuge.
- Incubate the mixture at 70°C for 5 minutes, chill on ice.
- Add 4µl of 5x reaction buffer.
- Add 1µl RiboLock ribonuclease inhibitor (20u/µl).
- Add 2µl of 10mM dNTP mix. Mix gently.
- Incubate at 37°C for 5 minutes.
- Add 1µl RevertAid H Minus M-MuLV Reverse Transcriptase (200u/µl).
- Incubate the mixture at 42°C for 60min.
- Stop the reaction by heating at 70°C for 10 mins. Chill on ice. The synthesized first strand cDNA can be used directly for amplification by PCR.

- Recipe for 25µl PCR mixture for cDNA amplification:
- Add 17µl distilled water
- Add 2.5µl of buffer (10x)
- Add 0.75 µl DMSO
- Add 0.75µl dNTP
- Add 1µl cDNA template
- Add 1.25µl of forward primer and 1.25µl of reverse primer
- Add 0.5µl DNA polymerase

PCR reaction
- Step 1: 94°C, 3 minutes
- Step 2: 94°C, 15 seconds
- Step 3: 55°C, 1 minutes.
- Step 4: 72°C, 45 seconds, repeat steps 2-4 30 times.
- Step 5: 72°C, 10 minutes

3.6.2.2. Ectoine Primers

Below are the primers used to amplify the *ect*A, *ect*B and *ect*C genes, responsible for ectoine synthesis. This helped us to positively identify the transformants. The positions in the DNA sequence where these primers bind are shown in **Appendix 2.**

***ect*A primers**

ectAf: 5'-ACGTCGTCCAACAGACGGTG-3'

ectAr: 5'-CTGTGAATGGACCAGCACGG-3'

***ect*B primers**

ectBf: 5'-ACGTGTTCGTTCATACTGTC-3'

ectBr: 5’-TGTTCTGACATAACAGCAGC-3’

***ect*C primers**

ectCf: 5’-GGTACATGGGATTCAACACG-3’

ectCr: 5’-TCATCATGAACTTCACGACC-3’

N.B: f is denoting forward primer, while r is denoting reverse primer.

3.6.3. Salt tolerance test

To test for salt tolerance, 50 mL of transformant cultures, in the exponential phase, were poured into different salt solution concentrations. The final NaCl concentration of the mixtures were 0%, 0.5% and 1% respectively.

The mixtures were left for 24 hours in continuous light before visually analysing the results by observing the colour differences.

4.0. Results

4.1. Construction of the transformation vector

Part of the original plan of this project was to ligate the optimized *ect*B gene into a vector containing *ect*A/C gene sequences. This vector was made by Victoria Holck and is approximately 9800 bp (Holck, 2014). It was constructed by inserting the optimized *ect*C gene fragment into the pCrc_32_OectA plasmid (Lunde, 2012) which contained an inserted optimized *ect*A gene. The cloning steps are shown in **Figure 8.**

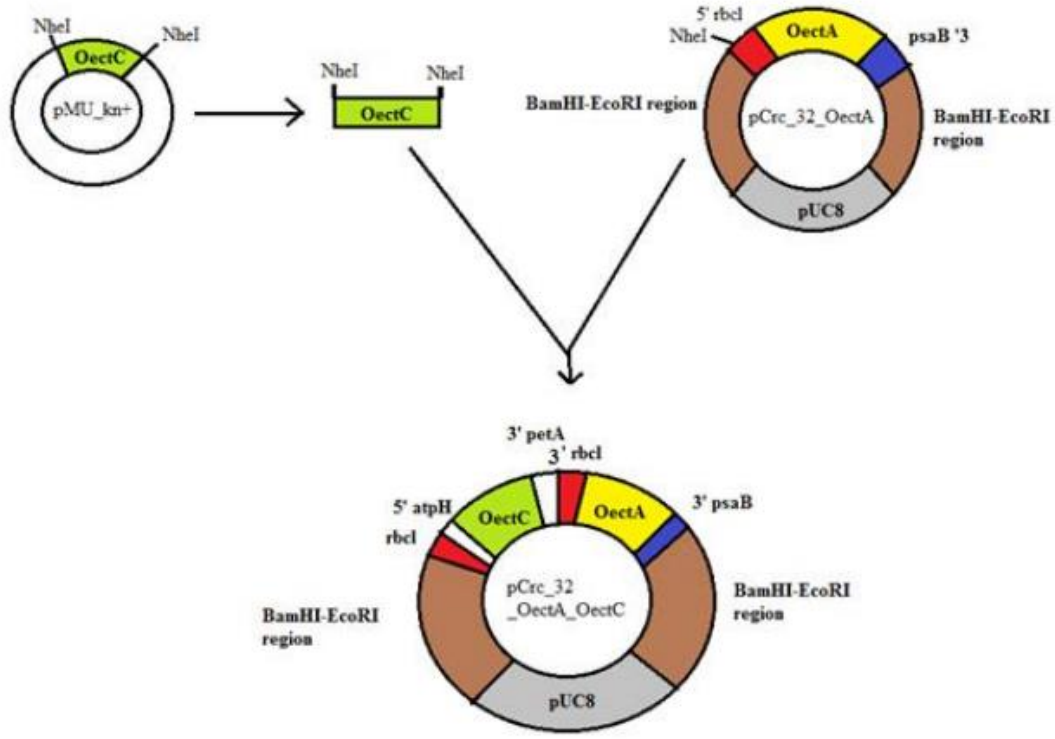


Figure 8. The pCrc_32_OectA_OectC plasmid was constructed by inserting the *ect*C gene fragment from the pMU_kn+ plasmid into the pCrc_32_OectA plasmid (Holck, 2014).

The final transformation vector was constructed in such a way that each gene (*ect*A, *ect*B and *ect*C) had its own promoter and transcription termination sequence.

The *ect*A gene was linked to the 5' region of the *rbcL* gene (this gene codes for the rubisco large subunit), and the 3' region of the *psaB* gene (codes for subunit B of photosystem I).

The *ect*B gene was linked to the 5' region of the *psbD* gene (codes for a photosystem II subunit) and the 3' region of the *rbcL* gene.

The *ect*C gene was linked to the 5' region of the *atpH* gene (this gene codes for an ATPase III subunit), and the 3' region of the *petA* gene (codes for a cytochrome f).

4.1.1. Difficulties with the insertion of *ect*B

Our plan was to insert the *ect*B gene fragment of approximately 1600bp into the pCrc_32_OectA_OectC plasmid (9800 bp), bringing the expected total to approximately 11,400 bp.

In this procedure, the pCrc_32_OectA_OectC plasmid was digested using the XhoI enzyme and later dephosphorylated using calf intestinal phosphatase, to avoid religation.

The pMA_RQ plasmid shown in **Figure 9**, containing *ectB* gene was amplified in *E. coli* TB1 cells and digested with SalI. SalI released the *ect*B segment from the pMA_RQ plasmid.

After agarose gel electrophoresis, the segment containing *ect*B was excised from the gel and purified. The purified segment was then ligated into pCrc_32_OectA_OectC. XhoI and SalI have compatible restriction sites, hence their products after digestion are able to ligate to each other. After ligation, the mixture was cloned into competent *E. coli* TB1 cells. Plasmid DNA was isolated from transformants and digested with PstI and analysed by agarose gel. **Figure 10** shows the schematic diagram of the final product we expected to have after cloning the *ect*B gene into the pCrc_32_OectA_OectC plasmid.

This however proved to be unsuccessful as shown in **Figure 11** from our plasmid minipreparations.

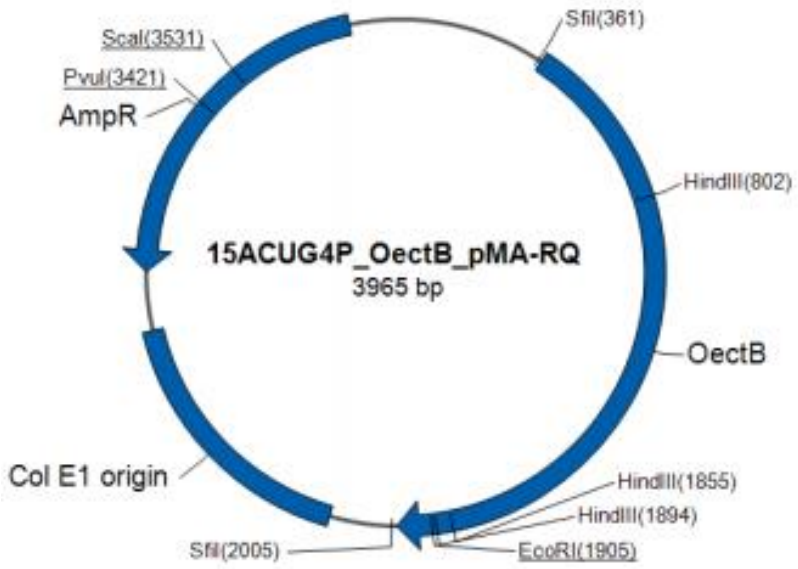


Figure 9. The pMA_RQ plasmid (check section **3.4** how it was constructed) containing the *ect*B gene.

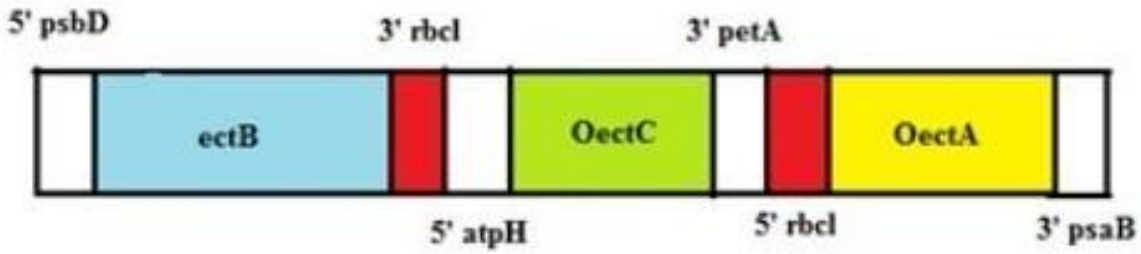


Figure 10. The final product we expected to have after cloning the *ect*B gene into the pCrc_32_OectA_OectC plasmid.

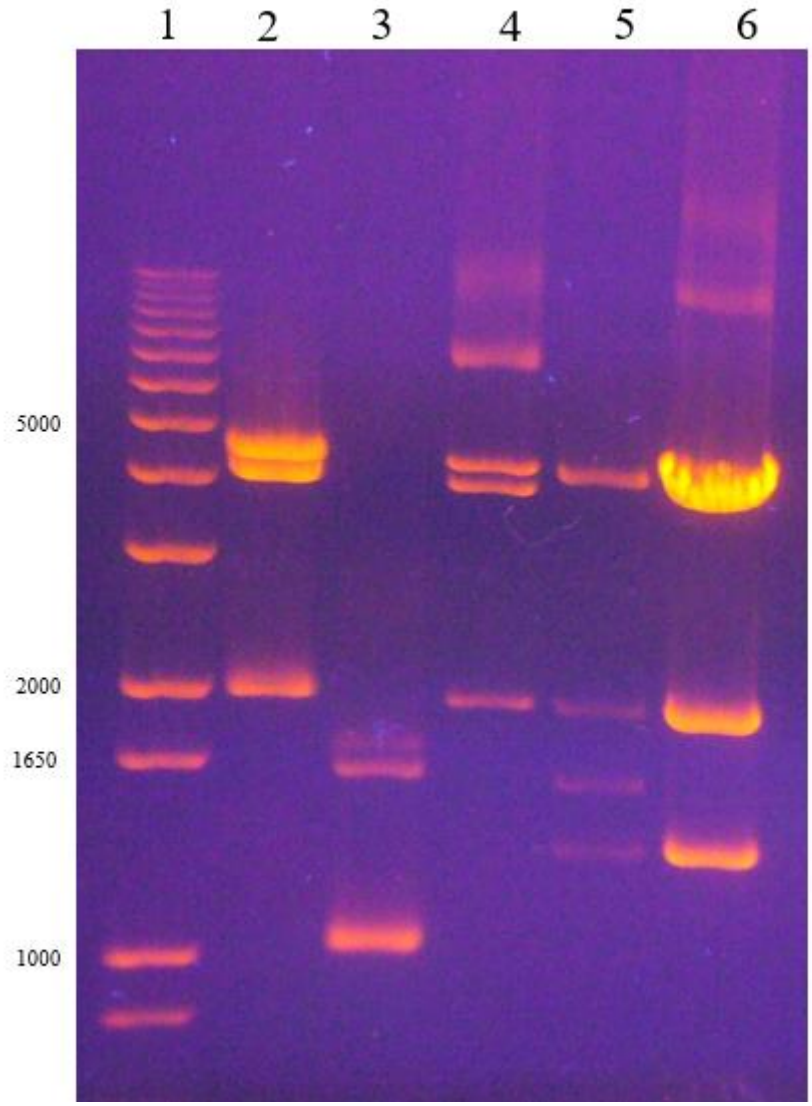


Figure 11. Miniprep results used to verify if we had successfully managed to insert the *ect*B gene fragment into the pCrc_32_OectA_OectC plasmid. The plasmid was digested with PstI to analyse the transformants for insert size. Lane 1 is the 1Kb ladder. Lane 2 is linearized pCrc_32_OectA_OectC plasmid (control). Lane 3 to 6 are plasmid DNA from transformants. Lane 3 has very few fragments to make approximately 11400 bp, the expected total. Lane 4 has the same fragments as the control. The fragments in lane 5 and 6 are not adding up to 11,400 bp. This suggests that our attempt to ligate *ect*B with the pCrc_32_OectA_OectC vector failed.

4.1.2. Troubleshooting and eventual successful ligation

We made several attempts to insert the *ect*B gene into the *ect*A/C genes construct without any success. During this time, we made several troubleshooting attempts, which included varying the molar ratio between the vector and the insert, reducing any possible vector background, changing the buffers and minimizing UV exposure of the excised *ect*B fragment from the gel until we successfully managed to insert the *ect*B gene into the pCrc_32_OectA_OectC plasmid as shown in **Figure 12.**

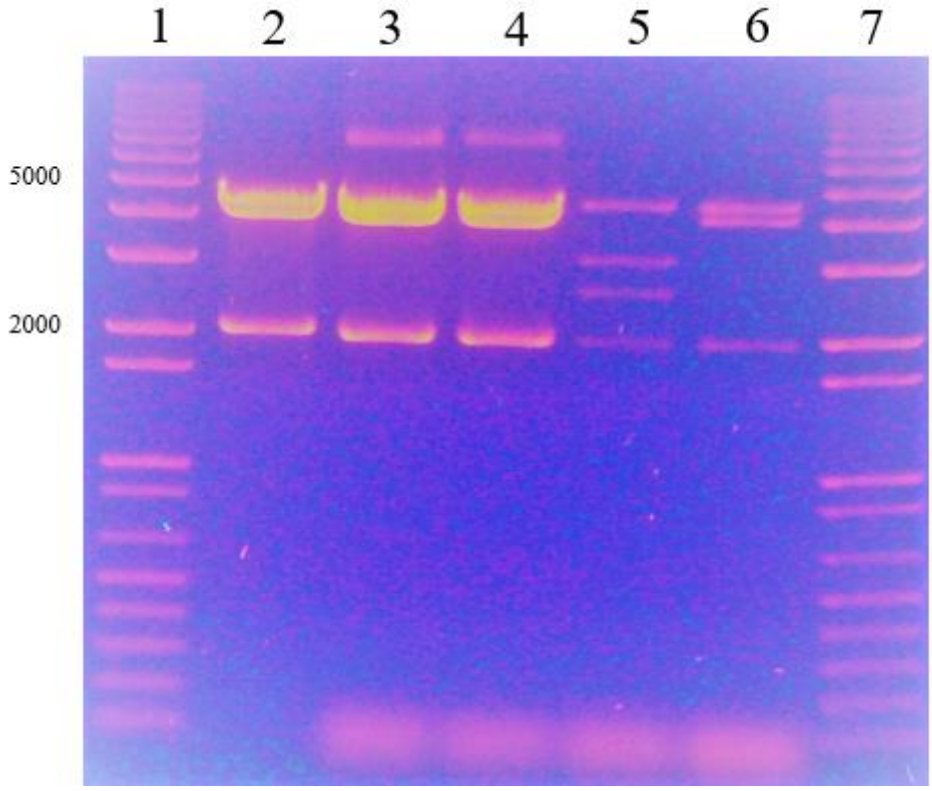


Figure 12. The restriction enzyme digest of the pCrc_32_OectA_OectC plasmid cut with PstI. Lane 1 and 7 are the 1Kb ladders. Lane 2 is the control plasmid (containing *ect*A/C), while lanes 3 to 6 are plasmid DNA from the transformants. Lane 3,4 and 6 have the same fragments compared to the control, hence had no insertion. Lane 5 shows a transformant with extra inserts (2600 bp and 3200 bp), suggesting *ect*B insertion.

We made a maxipreparation of this transformant (lane 5), after which we sent the sample (maxiprep sample) to Eurofins in Germany for DNA sequencing.

The sequencing helped us to confirm the insertion of the *ect*B gene into the *ect*A/C genes construct and its orientation.

4.2. Microprojectile bombardment and selection of transformants

Gold particles were coated with the transformation vector carrying *ect*A, *ect*B and *ect*C genes and bombarded onto plates of agar where a *Chlamydomonas* photosynthesis deficient mutant (atpB deletion mutant) was plated. The vector (plasmid) contains a full length *atpB* gene, which can complement the mutated atpB gene in the mutant by homologous recombination.

After bombardment, the cells were transferred to high salt (HS) plates for photosynthetic selection. After 2-3 weeks, the transformants grew on the plates as a result of the *atpB* gene uptake. Those which were unable to take up the *atpB* gene died because they were photosynthesis deficient. We had 50 transformants in total.

Figure 13 below shows the transformants from this part of the experiment.

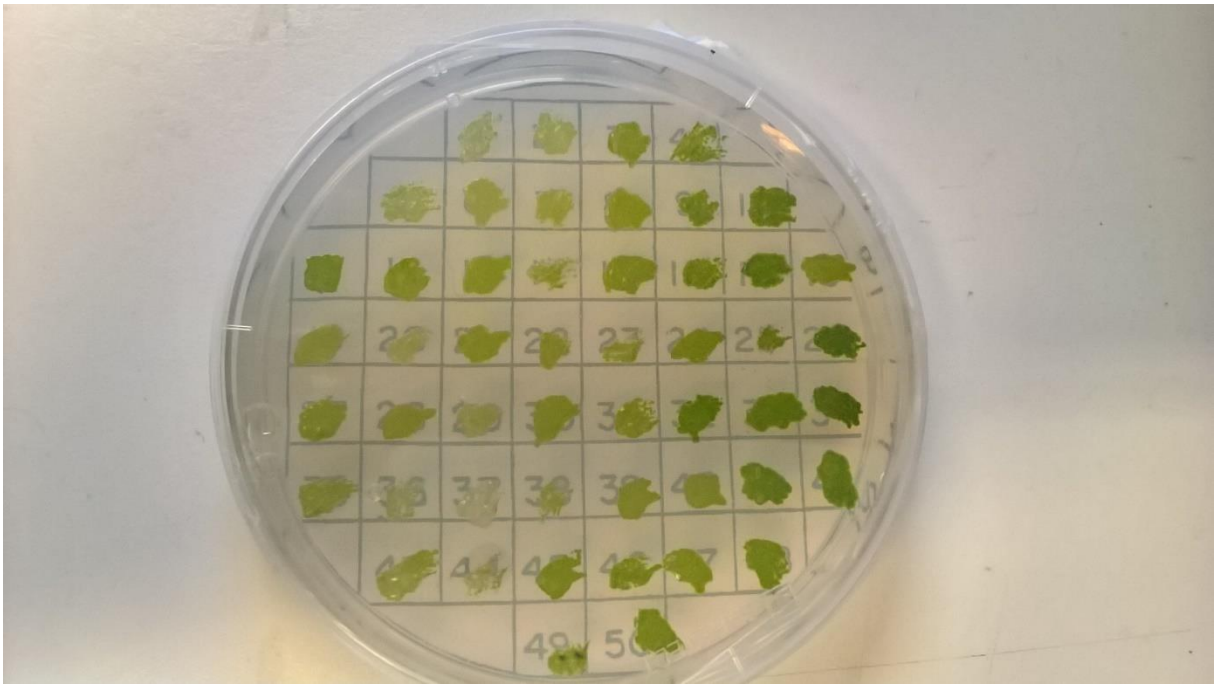


Figure 13. The transformants picked for further analysis. These transformants grew as a result of the *atpB* gene uptake.

DNA was isolated from 11 transformants and PCR, using the *ect*A, *ect*B and *ect*C genes primers and gel electrophoresis were done in order to identify transformants which had all the three genes (*ect*A, *ect*B and *ect*C).

One of the analyzed 11 transformants, transformant 21 harbored all the three genes. Because of time limits, no other transformants were analyzed.

Figure 14, below shows the gel electrophoresis results of transformant 21. Plasmid DNA from the *ect*ABC construct was used as control. The part of the DNA sequence where the primers bind is shown in **Appendix 2.**

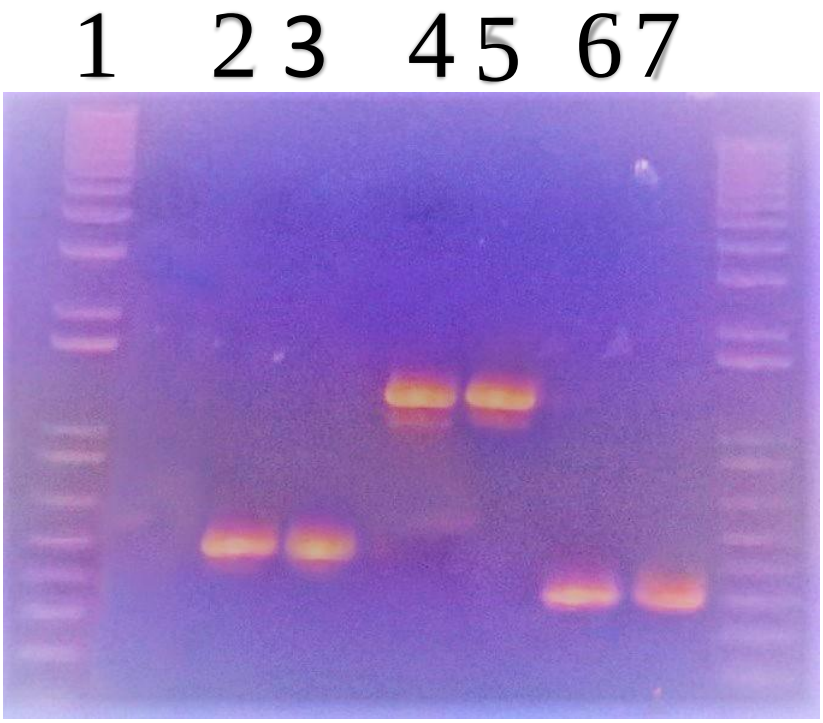


Figure 14. Gel electrophoresis results used to identify *ect*ABC positive transformants. PCR using the *ect*A, *ect*B and *ect*C primers were used before running the PCR products on the gel. Lane 1 is the 1kb plus ladder, lane 2 is *ect*A (480 bp), lane 3 is the standard (we used the *ect*ABC construct) which was used as the standard for *ect*A. Lane 4 is *ect*B (1230), lane 5 is the standard (*ect*ABC construct), lane 6 is *ect*C (305 bp) and lane 7 is the standard (*ect*ABC construct).

4.3. Reverse transcription to detect *ect*A, *ect*B and *ect*C genes expression.

RNA was isolated from transformant 21 according to the recommended lab protocol (see section 3.5.11). In order to eliminate any possible contamination of DNA, the RNA sample was treated with DNase following New England Biolab`s (DNase kit supplier) recommended protocol. We run the DNase treated RNA samples on PCR with *ect*A, *ect*B and *ect*C genes primers. When the PCR products were checked on agarose gel, no amplification was detected. This helped us to rule out any DNA contamination.

The RNA was then reverse transcribed and the products amplified by PCR using *ect*A, *ect*B and *ect*C genes primers (see section 3.6.2.2). The PCR products were then run on agarose gel (**Figure 15**). PCR products from the *ect*ABC construct was used as standard.

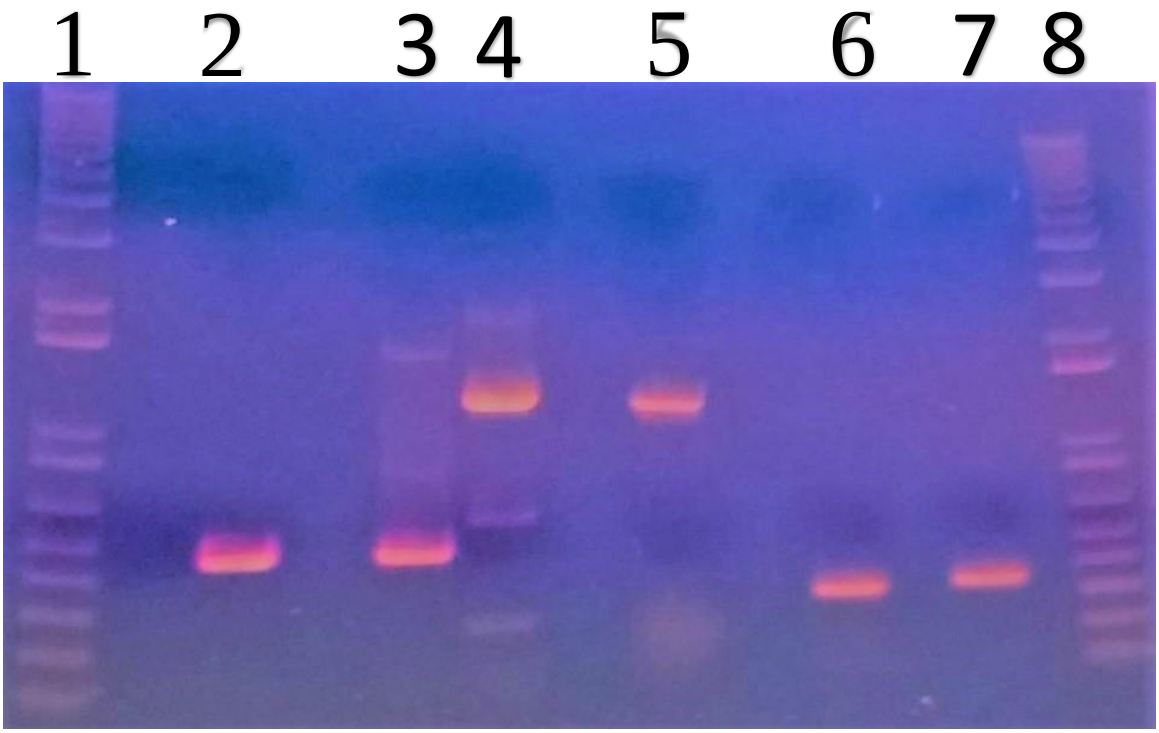


Figure 15. Gel results of the *ect*ABC DNA construct PCR products and the cDNA PCR products. This was done to detect the gene expression of the *ect*A, *ect*B and *ect*C genes. Lane 1 and 8 are the 1 kb plus DNA ladders, Lane 2: contains *ect*A cDNA (approximately 500 bp), lane 3: contains the standard (*ect*ABC DNA construct PCR product), Lane 4: contains *ect*B cDNA (approximately 1300 bp), Lane 5: contains the standard (*ect*ABC DNA construct PCR products). Lane 6 contains *ect*C cDNA (approximately 350 bp) and lane 7: contains the standard (*ect*ABC DNA construct PCR product). The size

correlation between the standards and the cDNA suggests that the three genes (*ect*A, *ect*B and *ect*C) were being expressed.

4.4. Salt tolerance test

In order to see if the transformant showed increased salt tolerance we grew it in different salt concentrations. In the first tube, there was 0% salt concentration, the second tube had 0.5% salt concentration and the last one had 1% salt concentration. The transformant (in three different salt concentrations) was grown for 72 hours in continuous light. The colours of the transformants were not very different as shown in **Figure 16**, although there was a noticeable slight reduction in the colour intensity as the salt concentration increased. This suggest that the transformants had obtained increased salt tolerance.

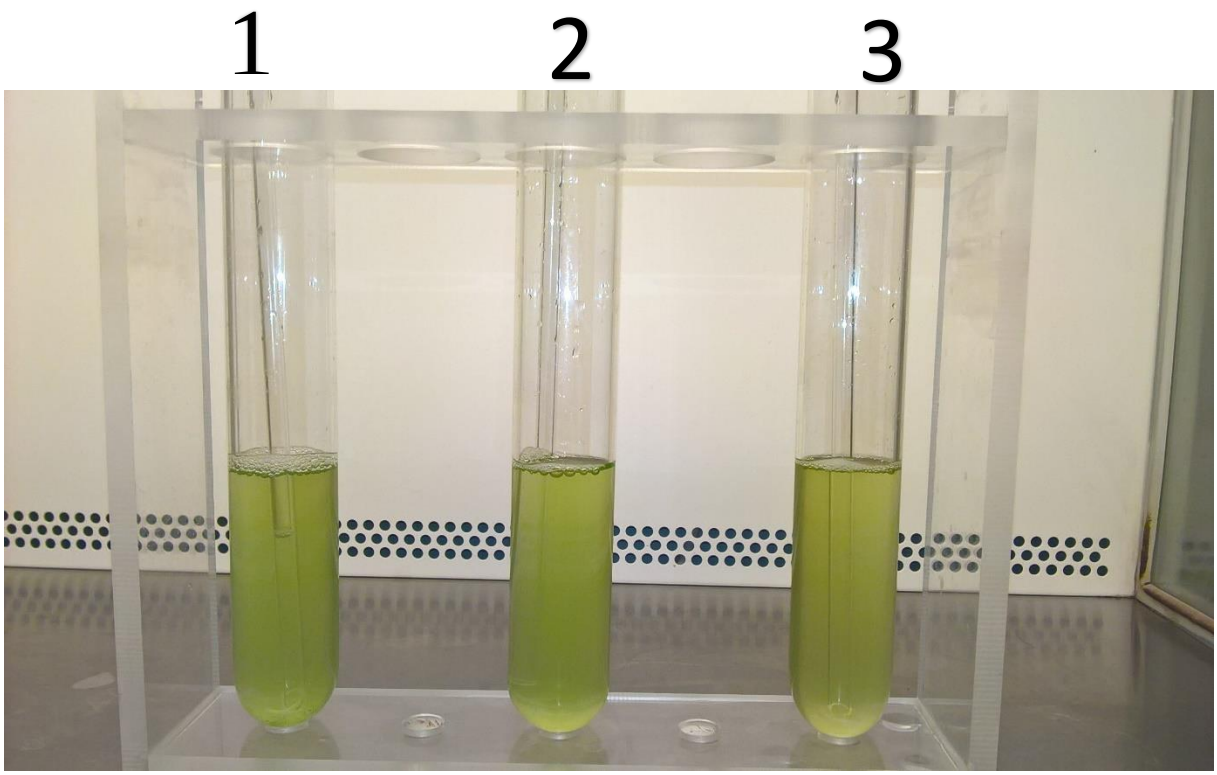


Figure 16. *Chlamydomonas* transformants in NaCl solution. Tube 1 contained 0% NaCl concentration. Tube 2 contained 0.5% NaCl concentration and tube 3 had 1% NaCl concentration.

5. Discussion

The *ect*A, *ect*B and *ect*C genes from *Pseudomonas stutzeri* were transformed into the *Chlamydomonas* chloroplast. The genes were inserted into the chloroplast genome in such a way that each individual gene had its own promoter and termination sequence.

The main aim was to synthesize ectoine and determine if the production of this compound could increase the salt tolerance of the transformants. PCR and agarose gel electrophoresis were used to positively identify the transformants containing all the three genes. Our observation based on the transformant we worked with indicated that we obtained increased salt tolerance.

The rest of this section contains an in-depth discussion of the results.

5.1. Difficulties encountered during the insertion of ectB gene

Among the main goals of this project was to insert an optimized *ect*B gene in the pCrc_32_OectA_OectC plasmid containing optimized *ect*A and *ect*C genes. This plasmid was constructed by inserting the optimized *ect*C gene fragment into the pCrc_32_OectA plasmid which contained an inserted optimized *ect*A gene. The pCrc_32_OectA plasmid contains the *atpB* gene which is cardinal in photosynthetic selection when introduced into *atpB* gene deficient *Chlamydomonas reinhardtii* mutants (Holck, 2014).

The previous students who worked on this project, Anders Lunde, Victoria Teigland, Holck and Nafisa Batool Afzal (Lunde, 2012), (Holck, 2014), (Afzal, 2014), didn't detect any increased salt tolerance. The fact that they didn't optimize all the genes gave us a reason to set up a study in order to find out if the lack of increased tolerance in the transformants was due to not optimizing all the genes. As a result it was the goal of this experiment to have all the genes in an optimized form.

However, several attempts to insert *ect*B in the *ect*A/C construct initially proved unsuccessful.

We made several troubleshooting attempts like varying the molar ratio of vector to insert, changing the batch of restriction enzymes, changing buffers, redoing CIP inactivation, reducing any possible vector background and minimizing UV exposure of the excised *ect*B fragment from the gel until we successfully

managed to insert the *ect*B gene into the pCrc_32_OectA_OectC construct. Looking at the number of troubleshooting attempts we made before we successfully managed to insert the *ect*B gene into the vector, it is hard to pinpoint exactly where we were getting it wrong. The likely explanations are that our initial, vector to insert size ratios were not ideal for proper ligation. Another explanation could be that our vector preparation was not done very well resulting into damaged or undigested vectors being through into the ligation, hence reducing the transformation efficiency.

We also changed the batches of the competent cells we were using on several occasions, it could be that our initial batches were not very effective.

Lastly, we can`t rule out the possibility of UV light damaging our plasmid in our initial attempts. In the end we tried as much as possible to minimize the damage by exposing the plasmid to UV for the shortest possible time.

5.2. Expressing codon optimized *ect*A, *ect*B and *ect*C genes in *Chlamydomonas reinhardtii* and why some transformants didn't contain all the three genes.

We used microprojectile bombardment to introduce the vector carrying the *ect*A, *ect*B and *ect*C into *Chlamydomonas reinhardtii.* The vector contained a full length *atpB* gene, which complemented the mutated *atpB* gene in *Chlamydomonas reinhardtii.* This resulted into the transformants. We analyzed several transformants for the inserted genes before we chose one transformant for analysis. We were supposed to have many transformants to analyze, but the limited time frame on this project forced us to stick to the one transformant with all the genes we found after analysing several transformants. The transformant (section 4.2) showed that the *ect*A, *ect*B and *ect*C genes were stably integrated into *Chlamydomonas reinhardtii.* The reason why the other transformants didn't contain all the genes may be due to the chloroplast chromosomes` ability to integrate a complete *atpB* gene without incorporating all the *ect*ABC genes.

5.3. Detecting *ect*A, *ect*B and the *ect*C genes expression

To ascertain the expression of the *ect*A, *ect*B and the *ect*C genes. We isolated RNA from the transformants and reverse transcribed it into cDNA. The positive results we got in section 4.3 confirmed the expression of the *ect*ABC genes, although this does not confirm the production of ectoine which needed an independent verification process, for example by using mass spectrometry.

5.4. Salt Tolerance test

To access if there was any increase in salt tolerance of the transformant. The transformant was grown in different salt concentration of 0% NaCl, 0.5% NaCl and 1% NaCl. There was a marked increase in tolerance of the transformant. The colour differences in the three tubes were minimal, although the intensity reduced as the salt concentration increased.

When we looked at the cells under the microscope, they were motile at 0 % NaCl and immotile at 0.5 % and 1 % NaCl respectively. We further observed that the cells formed clumps at higher salt concentrations (0.5 % and 1 %), which is an indication of reduced viability as the salt concentration increased.

This experiment indicated ectoine accumulation and increased salt tolerance.

6. Conclusion

The *ect*ABC genes were successfully cloned into the *Chlamydomonas reinhardtii* chloroplast. This experiment showed increased tolerance to salt in the *Chlamydomonas* transformant we analyzed. However further work is needed on this project. There is need to work on more samples in order to come to a firm conclusion.

7. Further work

There is need to increase the number of the *Chlamydomonas* chloroplast transformants with all the three genes (*ect*A, *ect*B and *ect*C) in the study. The specified period for this project coupled with other unforeseen circumstances limited our ability to explore other options. There were several transformants we didn't analyse due to limited time. Analysing many transformants could enable us come to a firm conclusion.

We didn't do any investigations to ascertain whether or not ectoine was produced by the transformants due to time factors. Hence there is need to analyse and quantify the amount of ectoine produced by using techniques like mass spectrometry and high-performance liquid chromatography (HPLC).

There is also need to investigate the possible bottlenecks which contributed to why some *Chlamydomonas* chloroplast transformants integrated complete *atpB* genes without incorporating all the *ect*ABC genes. Doing so might help increase the number of *Chlamydomonas* chloroplast transformants with all the three genes, which could qualify to be included in the study and also help reduce the time spent on screening.

The salt tolerance experiment could be redone using a non-ionic osmoticum, for example mannitol and sorbitol. This is because ions may inhibit growth of *Chlamydomonas* and possibly lead to cell clumping and death. A non-ionic osmoticum could eliminate this possibility.

Appendix 1. *ect*ABC genes in *Pseudomonas stutzeri*

Yellow: *ect*A

Grey: *ect*B

Green: *ect*C

TTAAGAGCG**GGAGCC**GGGAAAACTGCGGAATATATGGGCGTCACACTAGCGCCATATAT**T**
CCGGACTCGATCACAGAAGTCTTACGAATATCGAATCGCCCAGCGCGGCCTTATCCGCCC
AACCCCTTCGTTTGTCCCAGACGCCCCGCGCAAGCTACCAATCCGCCGCGCCAAGCACCG
CCACAGCCCTAGCCCGGCGCGGCTTTCAGCCAGGCAGACCGTCAGTCAGAGAACTTCTGA
CGCGCTCGGCGCTCGAACTTCCAGCCTGAATTCAAATTGCATTTCGGTGACAG**CTAAG**TT
GCCCGGCAAGCTGACCACCGCAATACACAGAAACATTCTGCGCGCCAGCATAGTTATCAT
GCGGGTTTCAGCGGCATATACAGCAACGCAAACTTCCCATATTCCGTTACATGGGTTGAG
AAACTTGTGCG**CAT****ATG**CCTACCCTAAAAAG**GAATTC**AATCAACAACCCCAAAGGCATTG
TTTTGAGTTTCCCCACCGTAATGCTCCGTCGCCCAACCGACGGCGACGGTTACAACCTTC
ATCAGCTGGTGGCGCGCTGCCAGCCCCTCGATACCAATTCGGTCTACTGCAACCTGCTGC
AGTGTTCCGATTTCGCTGACACCGCCATCGCCGCAGAGAACGCCCAAGGCGAGCTGGTCG
GTTTCATCTCGGGTTACCGCCCCCCTTCGCGGCCGGACACGCTGTTCGTCTGGCAGGTCG
CC**GTCGAC**AGTTCGATGCGCGGTCAGGGGCTGGCCCTGCGCATGCTGCTGGCACTGACCG
CCCGGGTCGCTCGCGAGTACGGCGTGCGTTACATGGAAACCACCATCTCGCCGGACAACG
GGGCGTCACAGGCGCTGTTCAAGCGGGCCTTCGACCGCCTCGATGCCAACTGCACGACGC
GCACGCTGTTTGCCCGCGACACGCATTTCGCCGGTCAGCACGAGGACGAGGTGCTCTACC
GCGCCGGCCCGTTCACCGTTTCCCA**TCTAGA**AGAA**GAGCTC**AAGGAGCACGCATGAAA
ACTTTTGAACTGAATGAATCCAGGGCTAGCAAACTAGTGCCGTTCCTTCCCCGTGGTCTTCA
AGCAGGCCCAGGGCGCCGAACTGGTCA**CTCAG**GACGGCAAGCGCTACATCGACTTCCTCGC
TGGTGCCGGCACGCTCAACTACGGGCACAACCACCCGGTGCTCAAGCAGGCGCTG**CTCGA**
GTACATCGAGAGCGACGGCATCACCCACGGCCTGGACATGTACACCGAAGCCAAGGAGCG
TTTCCTCGAAACCTTCAACCGGCTGATC**CTCGAG**CCGCGCGGCATGGGCGACTACCGCAT
GCAGTTCACCGGCCCGACCGGCACCAACGCGGTCGAGGCGGCGATGAAGCTGGCGCGCAA
GGTCACCGGGCGCAACAACATCATCAGTTTCACCAACGGCTTCCACGGCTGCAGCATTGG
CGCGCTGGCCGCCACCGGCAACCAGCATCACCGCGGCGGCTCCGGCATCGGCCTCACCGA
TGTCAGCCGCATGCCGTACGCCAACTATTTCGGCGACAAGACCAACACCATCGGCATGAT
GGACAAGCTGCTCTCCGACCCGTCCAGCGGGATCGACAAGCCCGCCGCGGTGATCGTCGA
GGTGGTCCAGGGCGAAGGCGGTCTGAACACAGCATCGGCCGAGTGGATGCGCAAG**CTCGA**

GAAGCTCTGCCGCAAGCACGAGATGCTGCTGATCGTCGATGACATCCAGGCCGGCTGCGG
CCGCACCGGGACTTTCTTCAGCTTCGAAGAGATGGGCATCCAGCCG**GATATC**GTCACGCT
GTCCAAGTCGCTGTCCGGCTACGGCCTGCCGTTCGCCATGGTGTTGCTGCGCCAAGAGCT
GGACCAGTGGAAGCCCGGCGAACACAACGGCACCTTCCGCGGCAACAACCATGCATTCGT
CACGGCGGCCGCGGCGGTCGAGCACTTCTGGCAGAACGACGCGTTCGCCAACAGCGTGAA
GGCCAAGGGCAAGCGCATCGCCGACGGCATGCAGCGCATCATCCGTCGCCACGGCCCGGA
TTCGCTGTTCCTCAAGGGCCGCGGGATGATGATCGGCATCAGCTGCCCCGATGGCGAGAT
TGCCGCCGCAGTGTGCCGCCACGCCTTCGAAAACGGCCTGGTGATCGAGACCAGCGGCGC
CCACAGCGAAGTGGTCAAGTGCCTCTGCCCGCTGATCATCAGCGATGAGCAGATCGACCA
GGCACTTTCCATCCTCGACAAGGCCTTTGCCGCCGTGATGAGCGAGCAGACCGAGAACC**A**
AGCTTC**CTGAG**GTATCCGCAATGATCGTCAGAACCCTCGCCGAGTGCGAAAAGACCGACC
GCAAGGTCCACAGCCAGACCGGCACCTGGGACAGCACGCGCATGCTGCTCAAGGACGACA
AGGTGGGATTCTCCTTCCACATCACCACCATCTACGCCGGCAGCGAGACGCACATCCACT
ACCAGAACCACTTCGAGTCGGTGTACTGCATCAGCGGCAATGGCGAGATCGAAACCATCG
CCGACGGCA**AGATCT**ACAAGATCGAGCCGGGCACGCTGTACGTGCTGGAGAAGCATGACG
AGCACCTGCTGCGCGGTGGCAGCGAAGACATGAAGCTGGCCTGCGTCTTCAACCCGCCGC
TCAACGGGCGCGAAGTGCATGACGAAAGCGGCGTCTATCCTCTGGAGGCCGAAACCGTCT
GATA**CCGGT**TTAACCGGGGCGGCCACCGCGCCGCCCTGCCATTACAAGAAAGGAGGTAAG
CGTGAACCCT

Appendix 2: The *ect*ABC genes in the *ect*ABC construct

Blue*: ectA*

Grey: *ect*B (in reverse)

Green: ectC (in reverse)

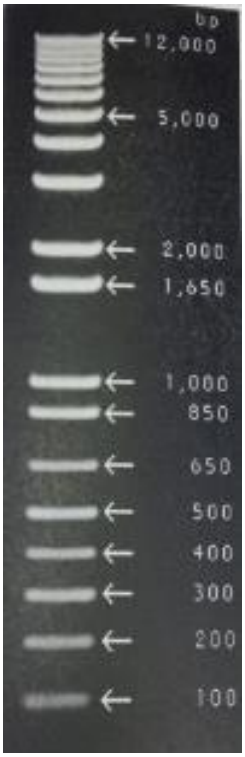
Red: promoter

Black: Primer positions

CACTATAGGGCGAATTGGCGGAAGGCCGTCAAGGCCGCATGTCGACTAATAATAAAAC
CTTTATTCATGGTTTATAATATAATAATTGTGATGACTATGCACAAAGCAGTTCTAGTCCC
ATATATATAACTATATATAACCCGTTTAAAGATTTATTTAAAAATATGTGTGTAAAAAATGC
TTATTTTTAATTTTATTTTATATAAGTTATAATATTAAATACACAATGATTAAAATTAAATAA
TAATAAATTTAACGTAACGATGAGTTGTTTTTTTATTTTGGAGATACACGCAATGAAAACT
TTTGAATTAAATGAATC**ACGTGTTCGTTCATACTGTC**GTTCATTCCCAGTTGTTTTCAAAC ***ect*Bf**
AAGCTCAAGGTGCTGAATTAGTTACTCAAGATGGTAAACGTTACATTGATTTCTTAGCTG
GTGCTGGTACATTAAACTACGGTCACAACCACCCAGTTTTAAAACAAGCTTTATTAGAAT
ACATTGAATCAGATGGTATTACACACGGTTTAGACATGTACACAGAAGCTAAAGAACGTT
TCTTAGAAACATTCAACCGTTTAATTTTAGAACCACGTGGTATGGGTGACTACCGTATGC
AATTCACAGGTCCAACAGGTACAAACGCTGTTGAAGCTGCTATGAAATTAGCTCGTAAA
GTTACAGGTCGTAACAACATTATTAGTTTCACAAACGGTTTCCACGGTTGTTCAATTGGT
GCTTTAGCTGCTACAGGTAACCAACATCACCGTGGTGGTTCAGGTATTGGTTTAACAGA
TGTTTCACGTATGCCATACGCTAACTATTTCGGTGACAAAACAAACACAATTGGTATGAT
GGACAAATTATTATCAGATCCATCATCAGGTATTGATAAACCAGCTGCTGTTATTGTTGA
AGTTGTTCAAGGTGAAGGTGGTTTAAACACAGCATCAGCTGAATGGATGCGTAAATTAG
AAAAATTATGTCGTAAACACGAAATGTTATTAATTGTTGATGACATTCAAGCTGGTTGTG
GTCGTACAGGTACTTTCTTCTCATTCGAAGAAATGGGTATTCAACCAGATATTGTTACAT
TATCAAAATCATTATCAGGTTACGGTTTACCATTCGCTATGGTTTTATTACGTCAAGAATT
AGACCAATGGAAACCAGGTGAACACAACGGTACATTCCGTGGTAACAACCATGCATTCG
TTACAGCTGCTGCTGCTGTTGAACACTTCTGGCAAAACGACGCTTTCGCTAACTCAGTTA
AAGCTAAAGGTAAACGTATTGCTGACGGTATGCAACGTATTATTCGTCGTCACGGTCCA
GATTCATTATTCTTAAAAGGTCGTGGTATGATGATTGGTATTTCATGTCCAGATGGTGAA
ATTGCTGCTGCAGTTTGTCGTCACGCTTTCGAAAACGGTTTAGTTATTGAAACATCAGGT
GCTCACTCAGAAGTTGTTAAATGTTTATGTCCATTAATTATTTCAGATGAACAAATTGACC
AAGCATTATCAATTTTAGACAAAGCTTTT**GCTGCTGTTATGTCAGAACA**AACAGAAAACC ***ect*Br**
AAGCTTCATAAGAATTCTTTTTATTTTTCATGATGTTTATGTGAATAGCATAAACATCGTTT
TTATTTTTATGGTGTTTAGGTTAAATACCTAAAGTCGACCTGGGCCTCATGGGCCTTCCG
CTCACTGCCCGCTTTCCAGTGATAAGACAAGTACATAAATTTGCTAGCCCGAAGGAGGC
AACTGCCACTAAAATTTATTTGCCCCAAGGGGACGTCCTTCGGAGTATAGTATATCAAAC
TTTAAAAATAACATACTAAAATTTGTAGGAGCTGCACAGCAGCCCTACAAAAAATTAAATA
CACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCGCATGCTAGCAGTTACCTCGC
CTATCGGCTAACCGTTTTGGGACGTCCTAATATAAATATTGGGATATTTAACCGTATAA
TGTTTTTCCACCATTGAAACAACCAAAGAATATAATATTCTTTGGTTGTTATCGATTTTA
TTGATTCATTTAGGAGGAAATACAATGATTGTTCGTACATTAGCTGAATGTGAAAAAAC

AGATCGTAAAGTTCATTCACAAACAGGTACATGGGATTCAACACGTATGTTATTAAAA *ect*Cf
GATGATAAAGTTGGTTTTTCATTTCATATTACAACAATTTATGCTGGTTCAGAAACACA
TATTCATTATCAAAATCATTTTGAATCAGTTTATTGTATTTCAGGTAATGGTGAAATTGA
AACAATTGCTGATGGTAAAATTTATAAAATTGAACCAGGTACATTATATGTTTTAGAAA
AACATGATGAACATTTATTACGTGGTGGTTCAGAAGATATGAAATTAGCTTGTGTTTTT
AATCCACCATTAAATGGTCGTGAAGTTCATGATGAATCAGGTGTTTATCCATTAGAAG *ect*Cr
CTGAAACAGTTTAATATTTAATTTTTTGTAGGGCTGCTGTGCAGCTCCTACAAATTTTA
GTATGTTATTTTTAAAGTTTGATATACTATACTCCGAAGGACGTCCCCTTGGGGCAAAT
AAATTTTAGTGGCAGTTGCCTCCTTCGGGCTAGCCTGGGCCTCATGGGCCTTCCTTTC
ACTGCCCGCTTTCCAGatattataTAAATATCCCAATATTTATATTAGGACGTCCCAAAACGG
TTAGCCGATAGGCGAGGTAACTGCTAGCTTACATTATTTTTTATTTCTAAATAtataatatATT
TAAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTTCCGGACAGATTATTTTAGGA
TCGTCAAAAGAAGTTACATTTATTTATACATATGCCTACATTAAAACGTAATTCAATTAA
CAACCCAAAAGGTATTGTTTTAAGTTTCCCAACAGTAATGTTACGTCGTCCAACAGAC *ect*Af
GGTGACGGTTACAACTTACATCAATTAGTTGCTCGTTGTCAACCATTAGATACAAATTC
AGTTTACTGTAACTTATTACAATGTTCAGATTTCGCTGACACAGCTATTGCTGCAGAAA
ACGCTCAAGGTGAATTAGTTGGTTTCATTTCAGGTTACCGTCCACCTTCACGTCCAGA
CACATTATTCGTTTGGCAAGTTGCTGTTGACAGTTCAATGCGTGGTCAAGGTTTAGCTT
TACGTATGTTATTAGCATTAACAGCTCGTGTTGCTCGTGAATACGGTGTTCGTTACATG
GAAACAACAATTTCACCAGACAACGGTGCTTCACAAGCTTTATTCAAACGTGCTTTCG
ACCGTTTAGATGCTAACTGTACAACACGTACATTATTTGCTCGTGACACACATTTCGCT
GGTCAACACGAAGACGAAGTTTTATACCGTGCTGG *ect*Ar
TCCATTCACAGTTTCACATTTAGAAGAAGAATTAAAAGAACACGCATAA

Appendix 3. 1kb Plus DNA ladder



1kb Plus DNA ladder

0.7 µg/lane

0.9 % agarose gel

Stained with ethidium bromide

Appendix 4. Solutions and recipes

Used in work with *E. coli*:

TEG (Tris-EDTA-Glucose)

25 mM Tris-HCl, pH 8.0, 10mM Na2-EDTA, 50mM glucose

Potassium acetate (3M potassium, 5M acetate) (1L)

294.42 g potassium acetate in 100 mL dH_2O. Add glacial acetic acid until a pH of 4.6. (About 40-50 % of final volume). Bring to 1 litre.

Lysogeny broth (LB) (1L)

10 g tryptone, 5 g yeast extract, 10 g NaCl. Add dH_2O to 1 litre.

For plates:

Add 15g agar per litre.

Sterilize in autoclave for 20 minutes.

If plates should contain any antibiotics, this should be added after autoclaving.

Ampicillin stock: In water 60 mg/mL. Use 1mL/ L.

The antibiotic stock solutions have to be sterilized by filtration through a 0.22 µm filter and later stored in small aliquots (1 ml) at -20^{o}C.

Tris-acetate EDTA buffer (TAE) 50x (1L)

242 g Tris base, 57.1 ml glacial acetic acid, 100 mL 0.5 M EDTA pH 8.0. Add dH2O to 1L. To make 1x, dilute in dH_2O.

Agarose gel loading buffer

0.25% bromphenhol blue, 0.25% xylene cyanol FF, 30% glycerol.

Media for growing *Chlamydomonas reinhardtii*:

HS (high salt) 1L

20 mL salt stock

20 mL phosphate stock

1 mL trace elements (Hunter)

HSHA (high salt high acetate) 1L

20 mL salt stock

20 mL phosphate stock

1 mL trace element (Hunter)

2.5 g potassium acetate.

For plates, add 15 g agar per litre.

Salt stock (50x) 500 mL

12.50 g NH_4Cl

0.50 g $MgSO_4$ x $7H_2O$

0.25 g $CaCl_2$ x $2H_2O$

Phosphate stock (50x) 500 mL

47 g K_2HPO_4 x

$3H_2O$ 18 g

KH_2PO_4

Sterilize in autoclave (1 litre for 20 minutes).

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