Expression of the bacterial *ectC* gene in the chloroplast of *Chlamydomonas reinhardtii*

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Per aspera ad astra

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Victoria Teigland Holck

Summary

The goal of this work was to express the three enzymes that catalyse the synthesis of ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) in the chloroplast of *Chlamydomonas reinhardtii*. Ectoine is an amino acid derivative that functions in many bacteria as compatible solute, helping the bacteria to survive and grow in highly saline environments. In addition, ectoine is used in cosmetic products, in the medical industry and for biotechnological purposes. There is also a potential relevance to agriculture, as synthesis of ectoine in cells of plants or algae could make them tolerant towards salt concentrations that would normally prohibit growth. There is an ongoing interest in developing efficient production systems for ectoine. Overexpression of ectoine in the chloroplast may lead to both an efficient and economical way to produce ectoine.

There are three bacterial genes, *ectA*, *ectB* and *ectC*, that encode the enzymes catalysing ectoine synthesis in bacteria. We wanted to insert the three genes into a suitable vector and transform the resulting construct into the chloroplast of *C. reinhardtii* in order to produce ectoine in *C. reinhardtii* cells and increase salt tolerance.

A non-photosynthetic *C. reinhardtii* mutant cell line was transformed by microprojectile bombardment with a plasmid vector containing a photosynthesis marker (the *atpB* gene) and the codon optimised transgenes *OectA* and *OectC*. Cloning of *ectB* was not possible in the time frame of this work.

Six *OectAC* chloroplast transformants (out of 30) were selected and screened for the presence of the *OectC* gene. Two positive transformants were further analysed for *ectC* mRNA accumulation. Very low levels of *ectC* transcripts could be detected in the two transformants, but no increase of salt tolerance was observed. It is concluded that *OectC* mRNA levels in the analysed transformant is too low for ectoine accumulation and that more transformants should be screened for the presence of the *OectAC* construct. In addition, insertion of the *ectB* gene could be performed in order to aid ectoine synthesis.

Abbreviations

atpB	-	Gene encoding the subunit of the CFl/CFo ATP synthase
atpH	-	Gene encoding an ATPase III subunit
bp	-	Base pair
BSA	-	Bovine serum albumin
Ca	-	Circa
CIP	-	Calf-intestinal alkaline phosphatase
Cm	-	Centimetre
DEPC	-	Diethylpyrocarbonate
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
DNase	-	Deoxyribonuclease
dNTP	-	Deoxynucleotide triphosphate
dsDNA	-	Double stranded deoxyribonucleic acid
e.g.	-	Exempli gratia (for example)
etc	-	Et cetera
g	-	Gram
g	-	Gravitational force
GFP	-	Green fluorescent protein
GRAS	-	Generally recognised as safe
HPLC	-	High pressure liquid chromatography
Kb	-	Kilo base pair
kDa	-	Kilo Dalton
L	-	Litre

M	-	Molar concentration
mA	-	Miliampère
Mb	-	Megabase pair
mg	-	Miligram
mL	-	Millilitre
mRNA	-	Messenger ribonucleic acid
MS	-	Mass spectrometry
N	-	Normality
NEB	-	New England Biolabs
ng	-	Nanogram
nm	-	Nanometer
OectA	-	Optimized ectA
OectC	-	Optimized ectC
petA	-	Gene encoding cytochrome f
pmol	-	Picomol
psaB	-	Gene encoding subunit B of photosystem I
psbD	-	Gene encoding photosystem II subunit
rbcL	-	Gene encoding rubisco large subunit
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
ssDNA	-	Single stranded deoxyribonucleic acid
TSP	-	Total soluble protein
UV-light	-	Ultraviolet light
UTR	-	Untranslated region

V	-	Volt
v/v	-	Volume by volume
w/v	-	Weight by volume
w/w	-	Weight by weight
µg	-	Microgram
µL	-	Microlitre
µCi	-	Microcurie

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1.0 Introduction

1.1 Salt tolerant organisms

1.1.1 Saline environments

Earth is a salty planet, with most of its water containing about 30 g of NaCl per litre (Flowers, 2004). According to DasSarma and DasSarma (2012) the oceans constitute approximately 99% of the biosphere for salt tolerant organisms. Small amounts of salt are required for all forms of life but for a long time it was believed that no life could exist in salt concentrations higher than 100 g NaCl per litre (10%). This is reflected by the names of many places throughout history e.g. in the name "the Death sea", a salt lake bordering Jordan to the east, and Palestine and Israel to the west, which on average contains 34% salt. Today it is known that the lake is inhabited by microorganisms (Melmer and Schwarz, 2009), and that environments with salt concentrations approaching saturation often are populated densely by microbial communities. A hyper-saline environment is defined as an environment containing salt concentrations in excess of seawater (3.5 % total dissolved salts). These hyper-saline environments are found all over the world in dry, costal and deep sea locations, in salt mines and in artificial salterns (Oren, 2002), (DasSarma and DasSarma, 2012).

1.1.2 Halophiles

Halophiles (from Greek; hal meaning sea or salt, and philos meaning love) flourish in saline environments. They are often classified as slightly, moderately, or extremely halophilic, depending on their requirement for NaCl. Optimal growth conditions for slightly halophilic organisms range from 0.2-0.85 M (1-5%) NaCl, and for moderately halophiles the range is 0.85-3.4 M (5-20%) NaCl. Extreme halophiles grow optimally with 3.4-5.1 M (20-35%) NaCl. In contrast, non-halophiles grow optimally in concentrations less than 0.2 M NaCl (< 1%) (DasSarma and DasSarma, 2012). Halophiles are found in all three domains of life, Bacteria, Archaea and Eukaryote (**Figure 1)** (Oren, 2008).

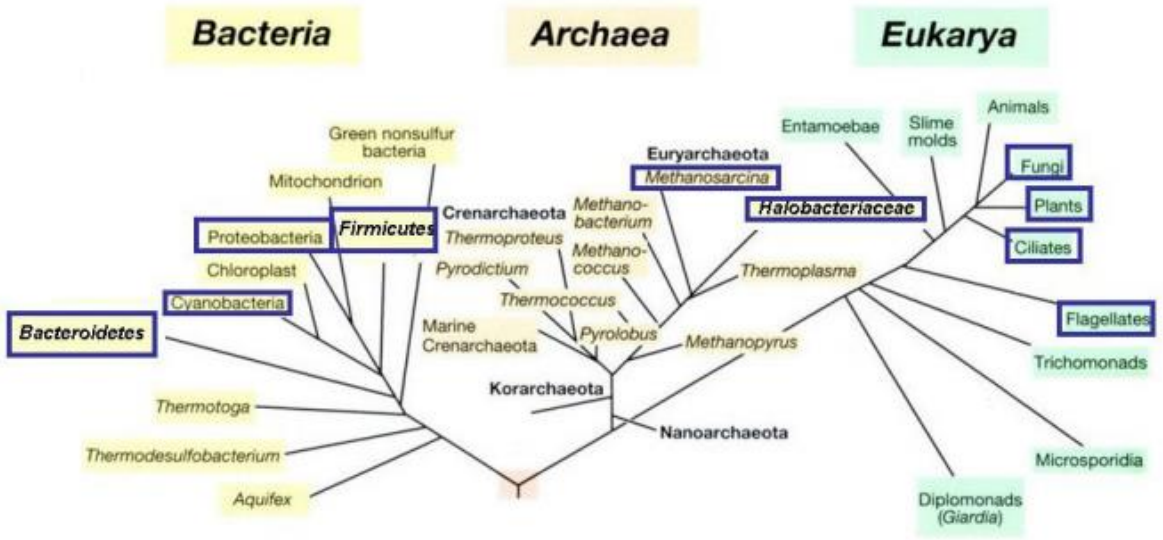

Figure 1: Distribution of halophilic microorganisms within the tree of life. Groups marked with bold blue boxes contain at least one halophilic representative. The tree is based on small subunit rRNA gene sequences. From Oren (2008).

1.1.3 Survival strategies

It is extremely important for halophilic microorganisms to balance their cytoplasm osmotically with the surroundings because cell membranes are permeable to water. **Figure 2** shows an example of what would happen in different surroundings if the microorganism did not possess any survival strategies. If cells lose water by osmotic processes, water can be actively transported inwards to compensate for the water lost. This is energetically unfavourable, and throughout evolution microorganisms have developed strategies to cope with high salt environments. The two main strategies are often called “salt-in” and “salt-out” (Oren, 2008).

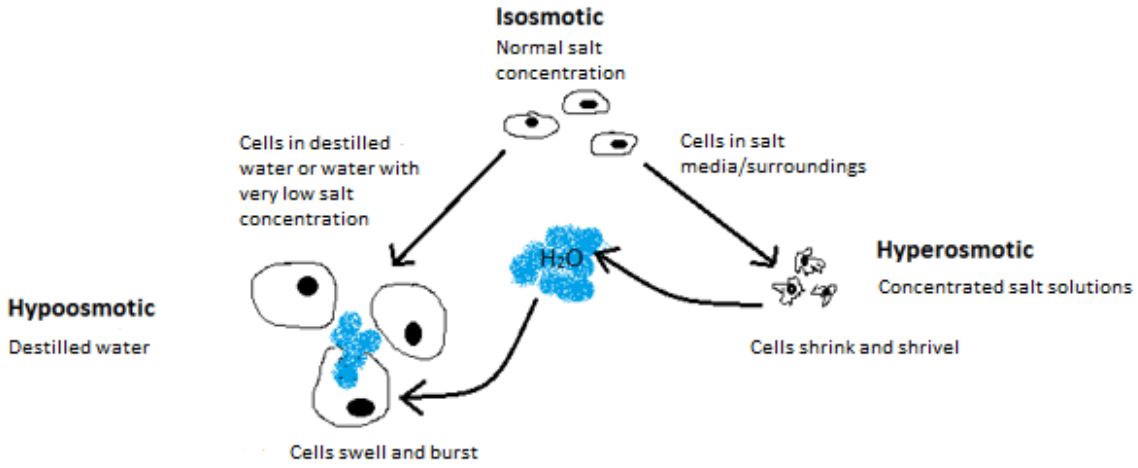

Figure 2: Overview of cells in high (hyperosmotic), normal, and low (hypoosmotic) salt concentrations. In both hyperosmotic and hypoosmotic environments cells will die if they do not possess any survival strategy.

1.1.4 Salt-in strategy

The salt-in strategy is based on the influx of ions from the environment. The cells accumulate potassium (K^+) and chlorine ions (Cl^-). Proteins in the saline cytoplasm need to be enriched in acidic amino acids (aspartate and glutamate) in order to remain functional. This is part of the adaptation of the microorganism's intracellular machinery, which will ensure that proteins will remain in their proper conformation, and continue to have normal activity in near-saturating salt concentrations. Most proteins of organisms using this strategy will denature when present in low salt environments, and therefore such microorganisms generally cannot survive in low salt media (Oren, 2008). Naturally, this strategy is most widespread amongst extreme halophile microorganisms (Empadinhas and da Costa, 2008). An overview of microorganisms using the salt-in strategy is given in **Figure 3**.

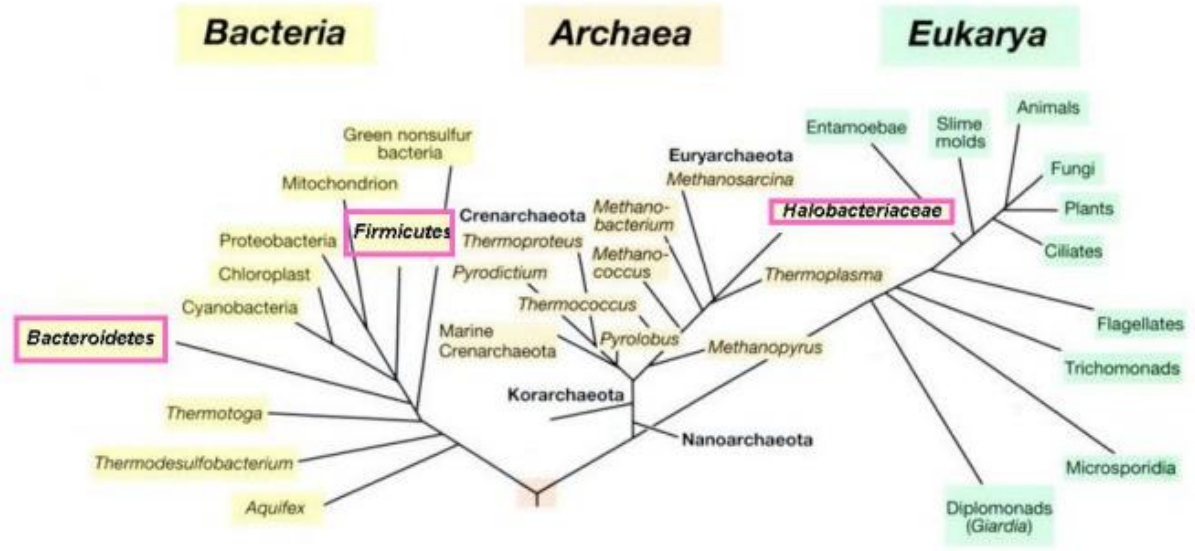

Figure 3: Distribution of microorganisms using the salt-in strategy. Groups marked with bold purple boxes contain microorganisms that use the salt-in strategy as their sole or main osmotic solute. From Oren (2008).

1.1.5 Salt-out strategy

The salt-out strategy is more common in nature than the salt-in strategy. This is because microorganisms using the salt-out strategy need very few adaptions of the cell proteome, and naturally, organisms using this strategy are adapted to a broad range of salt concentrations. The strategy involves both exclusion of salt from the cytoplasm and the synthesis and/or accumulation of small organic molecules (Oren, 2008). By accumulation of non-ionic molecules and/or Zwitterions (a natural molecule with both a positive and a negative charge) with low molecular weight, the decrease in water activity due to an increase in environmental salt conditions are balanced. These small molecules are named compatible solutes (DasSarma and DasSarma, 2012), (Melmer and Schwarz, 2009). **Figure 4** gives an overview of microorganisms using the salt-out strategy.

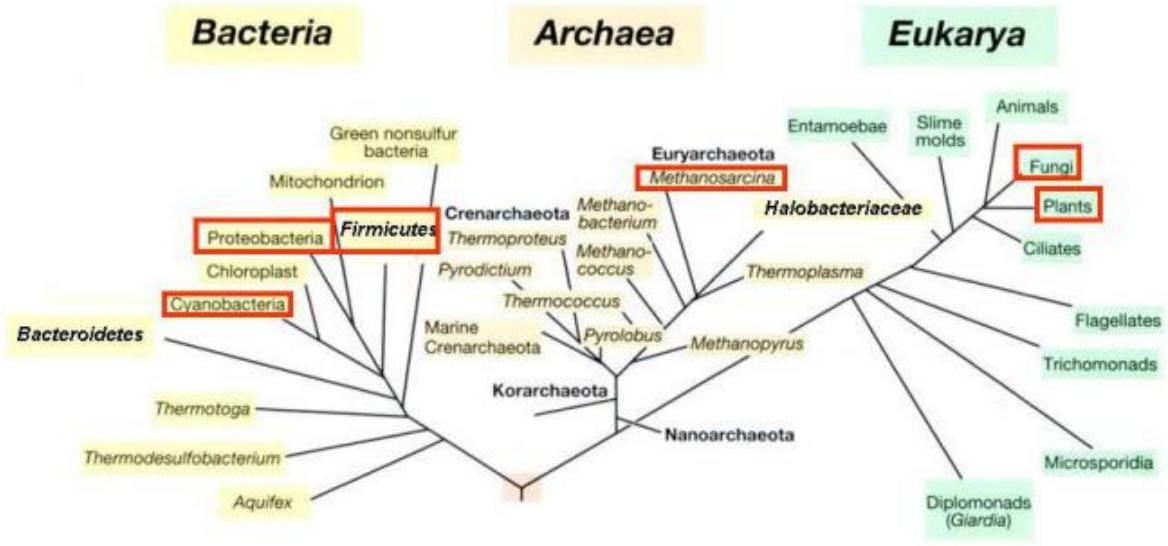

Figure 4: Distribution of microorganisms using the salt-out strategy. The groups marked with bold red boxes contain at least some halophilic representatives in which *de novo* synthesis and/or accumulation of organic solutes have been demonstrated. From Oren (2008).

1.1.6 Compatible solutes (organic osmolytes)

The compatible solutes (also called organic osmolytes), were so named because they do not inhibit the overall cellular pathways of the cell. Even though they can interfere with some enzymes, they are compatible with all of the cells functions. Compatible solutes are natural substances like alcohols, amino acids, sugars and derivatives of these compounds. Their main task is to help the cell regulate the osmotic pressure, and maintain protein stability (Arakawa and Timasheff, 1985). Compatible solutes act as chemical chaperones that either are produced by the cell itself or transported into the cell from the surroundings. The accumulation of compatible solutes helps the organism adapt to different environments e.g. freezing, high temperatures and salt stress, and they protect by maintaining the cell volume, the turgor pressure and the concentration of electrolytes (Roberts, 2005), (Kolp et al., 2006).

1.2 Ectoine

1.2.1 Discovery and characterization of ectoine

Ectoine was first found and characterized by Galinski et al. (1985) in the extremely halophilic phototrophic eubacterium, *Ectothiorodospira halochloris*, and hence the compound was named ectoine. Now we know that ectoine is widespread among both halophilic and halotolerant microorganisms (Melmer and Schwarz, 2009), and that it is one of the most commonly found osmolytes in nature (Zhu et al., 2014). The capacity to synthesize ectoine is most widespread among α- and ϒ-proteobacteria and actinobacteridae, although it has been observed also in a more limited number of β-, δ-, and ε-proteobacteria, firmicutes, and one plantomycete (Pastor et al., 2010). Ectoine can be considered either to be a heterocyclic amino acid or a partially hydrogenated pyrimidine derivate and is characterized as 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (Galinski et al., 1985). The structure of ectoine is shown in **Figure 5**, and the molecular formula is $C_6H_{10}N_2O_2$.

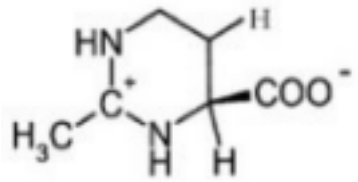

Figure 5: Structure of Ectoine. From Melmer and Schwarz (2009).

1.2.2 Syntheses of ectoine

The gene cluster *ectABC* is responsible for the synthesis of ectoine in bacteria. The cluster has been isolated and characterized from many eubacteria and archaea (Louis and Galinski, 1997), (Anbu Rajan et al., 2008). Ectoine is synthesized in three steps (**Figure 6**). The first substrate is an aspartate derivate called L-aspartate-β-semialdehyde. Glutamate and L-2,4-diaminobutyrat transaminase (encoded by *ectB*) are required in the first step to transform the L-aspartate-β-semialdehyde to L-2,4-diaminobutyrate. In step two, acetyl-CoA and L-2,4-diaminobutyrate acetyltransferase (encoded by *ectA*) are needed, and L-2,4-diamionobutyrate is transformed into N_2-acetyl-L-2,4-diaminobutyrate. In the final step the enzyme ectoine

synthase (encoded by *ectC*) transforms N_2-acetyl-L-2,4-diaminobutyrate to ectoine in a cyclic condensation reaction.

From ectoine, the synthesis can go on using 2-oxoglutarate, oxygen, Fe^{2+}, and ectoine hydroxylase (encoded by *ectD*) making hydroxyectoine. Hydroxyectoine also works as a compatible solute, but while ectoine, which is produced at once when the microorganism finds itself in a salty environment, hydroxyectoine is made primarily when the cultures enter the stationary growth phase (Bursy et al., 2007).

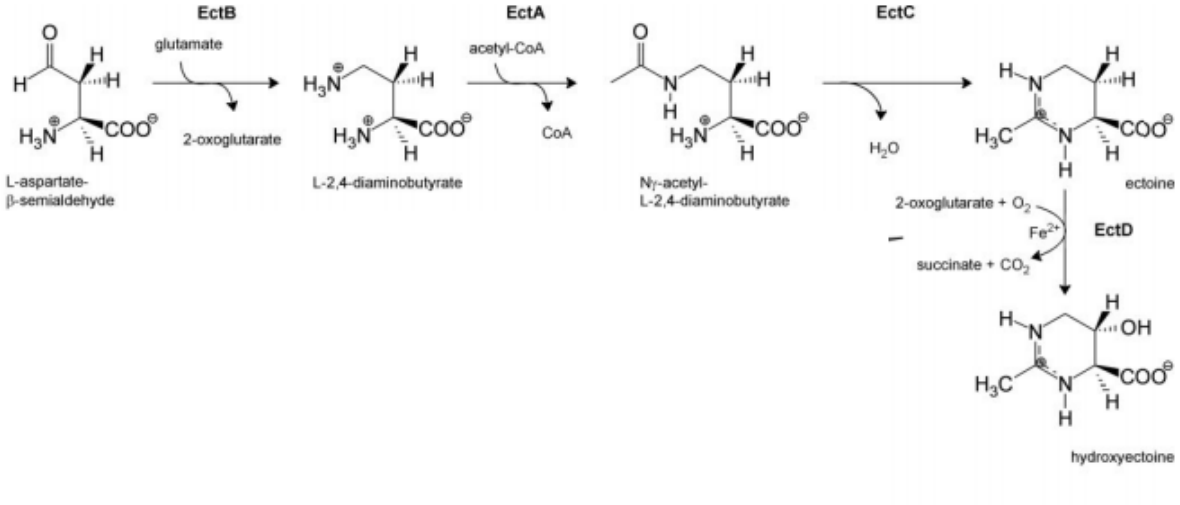

Figure 6: Pathway for the biosynthesis of ectoine and hydroxyectoine, from Bursy et al. (2007).

The two last steps in the ectoine syntheses pathway seems to be the most important once, since they are unique and do not participate in any other syntheses that we know of. The first step however from L-aspartate-β-semialdehyd to L-2,4-diaminobutyrate accrues naturally in *C. reinhardtii* because of a endogenous *ectB* gene in the syntheses pathway of the amino acids arginine and proline (**Figure 7**).

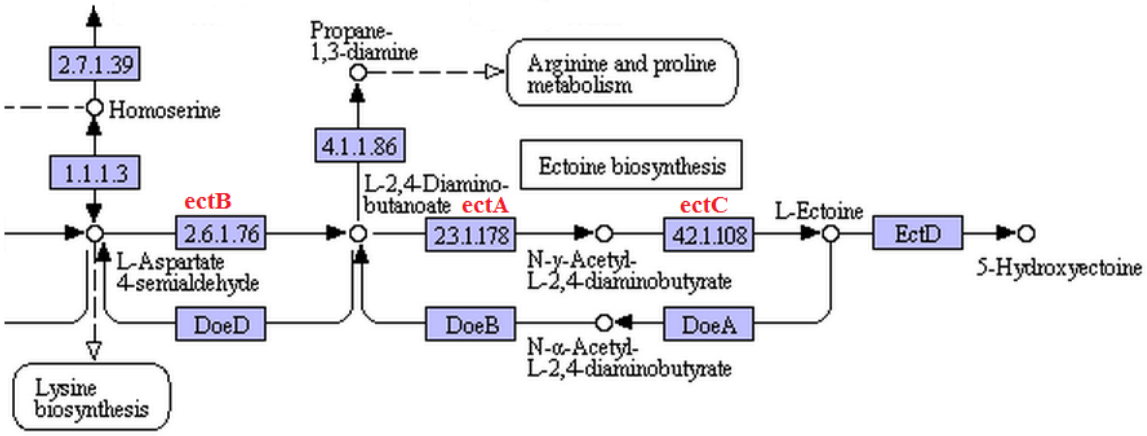

Figure 7: The biosynthetic pathway of ectoine. *ectA* encodes L-2,4-diaminobutyrate acetyltransferase, and *ectC* encodes ectoine synthase. The enzyme L-2,4-diaminobutyrate transaminase (encoded by *ectB*) occur naturally in the syntheses of the amino acids arginine and proline. Adapted from KEGG (http://www.genome.jp/dbget-bin/www_bget?pathway+ko00260).

1.2.3 Activity of ectoine and other compatible solutes

There are two main theories on how compatible solutes, like ectoine, works: the preferential exclusion model (**Figure 8**) and the water replacement hypothesis.

According to the preferential exclusion model, compatible solutes are excluded from having any direct protein surface contact since this can lead to unfavourable interactions, and to induce unfolding of proteins. This might provide the molecular basis for solute exclusion and, subsequently, the stabilization effect. A water layer forms between the compatible solutes and the proteins, forcing the proteins to occupy a smaller volume. This is called “wetting hydrophobic molecules”. Because the proteins wants to protect its hydrophobic parts from water, it stays in its native conformation. Unfolding would need additional energy, which is not thermodynamically favoured. When the compatible solutes do not react with the proteins, the catalytic activity remains unaffected (Pastor et al., 2010).

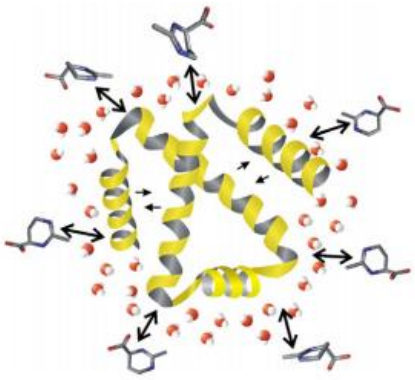

Figure 8: Stabilization mechanism of compatible solutes based on the preferential exclusion model. Small spheres represent water molecules and backbones represent compatible solutes (ectoine). The native conformation of the protein is favoured when compatible solutes are excluded from the protein surface. From Pastor et al. (2010).

The water replacement hypothesis is completely opposite to the preferential interaction model. This theory is based on the fact that many organisms can lose over 50% of cellular water and still return to full activity when the cell is rehydrated. Scientists supporting this hypothesis believe that water is replaced with compatible solutes that interacts with the protein surface and protects them from degradation.

Some scientists believe that both the water replacement model and the preferential interaction hypothesis are correct, and that replacement of water with compatible solutes only happens in extreme cases with very low water activities. The dilution model is the preferred model for the more diluted range of solute concentrations (Pastor et al., 2010).

1.2.4 Commercial use of ectoine

Today saline soil is a big challenge for agricultural production. Soil that contains a lot of NaCl and Na_2SO_4 is called saline soil. In contrast, soil that is dominated by Na_2CO_3 and $NaHCO_3$ is called alkaline soil. However, saline and alkaline soils are easily mixed together, and are often referred to as saline-alkaline soil. Salinization of land is becoming a big problem in many areas, especially considering the lack of fresh water in many places. The soil is often treated wrongly, with improper land irrigations and fertilization practices. In 2012 there were around ten million square kilometres of saline-alkaline soil in the world, a total of 7.6% of the earth's land area (Yu et al., 2012).

Increased salinity in the soil can disturb plants' ionic homeostasis, create a hyperosmotic state and eventually even lead to crop death. Generally, a soil salinity of 0.2% to 0.5% has a negative effect on plant growth; however, the surface of the soil in saline areas usually ranges from 0.6% to around 10%. Therefore, salt stress is a major agricultural concern and requires new methods to increase crops' salinity resistance and saline-alkali tolerance (Yu et al., 2012). If we could get "food plants" to express ectoine, many of the problems of growing in saline environments might be solved.

There are also many other reasons for wanting to produce high amounts of ectoine. Researchers have seen that ectoine could improve cell growth and utilization of glucose, and some researchers have reported ectoine to be a potential candidate for treating Alzheimer's disease. Ectoine is already used in creams because of its moisturizing effect which is even better than that of glycerol. It also protects the skin from harmful UV-A light, which could damage the cells in a number of different ways. Ectoine is proven to have anti-aging properties, and is licensed as a biomolecule stabilizer to be used in molecular biology applications. In addition, it is found to protect biomolecules from many variants of stress (protein stabilization, DNA stabilization, osmotic stress, thermostability, oxidation, detergents and degradation). Numerous other potential uses are still under investigation. The first cosmetic product containing ectoine was introduced on the market in 2001. In 2007 a nasal spray was launched, and became the first medical product containing ectoine. (Pastor et al., 2010), (Melmer and Schwarz, 2009).

1.2.5 Production of ectoine.

Since the demand for ectoine has increased over the last couple of years, a number of methods to improve ectoine production are being researched. Earlier, ectoine was either extracted from natural producers, which gave quite a low yield, or synthesized chemically. In the mid-nineties a fermentation process called "bacterial milking" was developed, and is still in use for ectoine production. The German company, Bitop AG, founded in 1993, is today the only large-scale commercial producer of ectoine, and they use the "bacterial milking" strategy for ectoine production. The bacterium *Halomonas elongate,* which produces both ectoine and hydroxyectoine, is grown in a hyperosmotic medium with 15% NaCl. After a while the bacteria are introduced to a hypoosmotic downshock of 3% NaCl. Now the bacteria do not need the compatible solutes any more, and release them in to the surrounding media. The

downshocked bacteria are then put back into the growing chamber at 15% NaCl to start producing compatible solutes again. The compatible solutes in the media are isolated and purified (Melmer and Schwarz, 2009).

There is today a lot of ongoing research to improve the "bacterial milking" process, and a new method is a cumbersome multi-step process (Melmer and Schwarz, 2009). Here the producer strain creates a mix of different compatible solutes, and ectoine needs to be purified from these. Also new genetic engineering experiments, and/or new wild type strains might be able to increase the quality and yield of ectoine. Biotechnological processes tend to be preferable because the use of organic solvents and toxic chemicals are avoided. Bacterial methods demand high amounts of nutrients and finely tuned culture conditions such as pH, aeration, and nutrient feeding during the operation of fermenters (Melmer and Schwarz, 2009), (Pastor et al., 2010).

1.2.6 *Pseudomonas stutzeri* and the *ectABC* gene cluster

P. stutzeri is a universal gram negative bacterium. It is often called universal because it is found in virtually all environments around the world, and therefore also has a widely diverse metabolism (Lalucat et al., 2006). Naturally, *P. stutzeri* produces the compatible solutes ectoine that makes the organism salt tolerant (Seip et al., 2011). Previous studies on transcriptional regulation revealed that the *ectABC* gene cluster is organized as an operon in almost all cases (Zhu et al., 2014). Appendix 3 shows the *ectABC* gene cluster organisation in *P. stutzeri*.

1.3 Chlamydomonas reinhardtii

1.3.1 *C. reinhardtii* as a model organism

C. reinhardtii is a unicellular freshwater eukaryotic green algae, 10 µm in diameter, whose lineage diverged from land plants over 1 billion years ago. *C. reinhardtii* has a simple life cycle, multiple mitochondria, two anterior flagella for motility and mating, and one single chloroplast (**Figure 9**) (Merchant et al., 2007). Because of its features, *C. reinhardtii* has been in use as a model organism for the past 50 years, and the whole genome of the algae has been sequenced. The linear mitochondrial genome of 15.8 kb was fully sequenced in 1993 (Vahrenholz et al., 1993). The single chloroplast genome which consists of 203 395 bp, was

fully sequenced in 2002. The chloroplast genome is divided by 21.2 kb inverted repeats into two single copy regions of about 80 kb, and contains 99 genes. 20% of the chloroplast genome is repetitive DNA (Maul et al., 2002). The alga has 17 linear chromosomes in the nucleus, and these were fully sequenced in 2007 (Merchant et al., 2007).

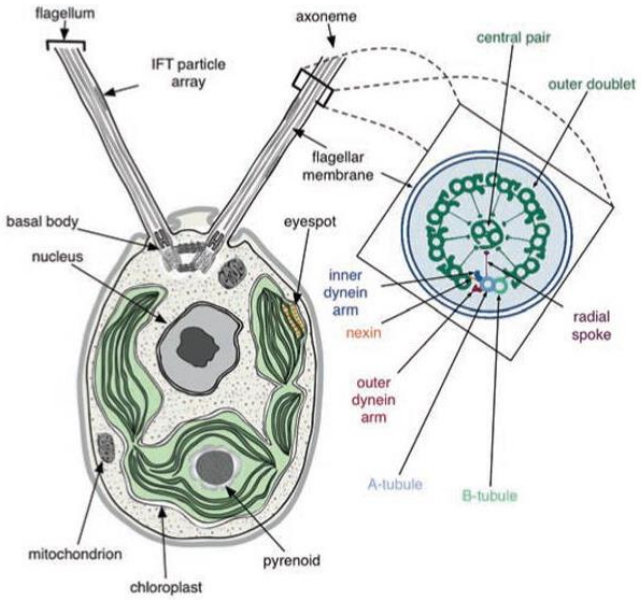

Figure 9: A schematic of a Chlamydomonas cell. From Merchant et al. (2007).

C. reinhardtii has several features that make it attractive as a model for expressing recombinant genes. The alga is easy to grow, either in agar or liquid media. The optimal growth temperature is between 20˚C and 36˚C, and it requires no additional vitamins or co-factors. *C. reinhardtii* grows best phototrophically (in constant light), and with extra supply of CO_2 (Potvin and Zhang, 2010). Transformation of the alga can be done with well-established techniques, and it takes from 2-6 weeks to generate a transformant cell line. The generation time under optimal conditions is about eight hours. *C. reinhardtii* is classified as a GRAS (generally recognized as safe) organism. It has no known virus or pathogens (Purton, 2007).

1.4 Transformation and optimization

1.4.1 Chloroplast transformation vs. nuclear transformation

Recombinant gene expression has been done in both nuclear, mitochondrial and chloroplast genomes of microalga, but expression good enough for commercial use has been achieved by chloroplast expression only. It has so far been very difficult to express transgenes in the nucleus. Reasons for this might be linked to positional effects; RNA silencing, chromatin structure and epigenetic effects. Because of the high expression level of genes in chloroplasts, they are generally chosen for transgene expression in microalgae. Surzycki et al. (2009) have observed protein yields varying from 0.88% to 20.9% of total soluble protein (TSP) when expressing a transgene in the chloroplast, but it has also been reported a yield as high as 45% TSP (Bock, 2007). Such high numbers only occur in rare cases, and the large majority of yields are around 5% of TSP and lower (Potvin and Zhang, 2010). Plastids and all their genetic information are usually inherited maternally, and are therefore excluded from pollen transmission. This means that scientists have an environmentally benign method for transgene expression. It is also possible to regulate the gene expression in chloroplasts by choosing appropriate combinations of plastid expression signals (e.g. promoters, Shine-Dalgarno sequences, 3´untranslated regions) (Bock, 2007).

1.4.2 *C. reinhardtii* chloroplast transformation

The first stable transformation of a *C. reinhardtii* chloroplast was actually the first stable transformation of any chloroplast ever done. This happened in 1988, and researchers found that chloroplasts worked very well for expressing recombinant products. Each chloroplast contains up to hundred copies of the plastome and most of the genes in the plastomes have roles in the photosynthetic pathway, which should therefore be highly expressed. Consequently, a transgene inserted into the plastome is amplified significantly compared to insertion of the same gene into the nuclear genome. DNA integration very often occurs through homologous recombination (**Figure 10**), and this allows very precise and predictable site-specific expression. Studies have also shown that transgenes expressed in the chloroplast are not subject to transcriptional or post-transcriptional gene silencing (Purton, 2007).

The downside of chloroplast transformation is that poor promoter activity and low mRNA stability can impact gene expression. Analysis of transgenic *C. reinhardtii* chloroplasts show sufficient heterologous mRNA accumulation to support high levels of protein synthesis

(Blowers et al., 1990), (Salvador et al., 1993). Another thing to keep in mind is that *C. reinhardtii* prefers monocistronic genes (Drapier et al., 1998).

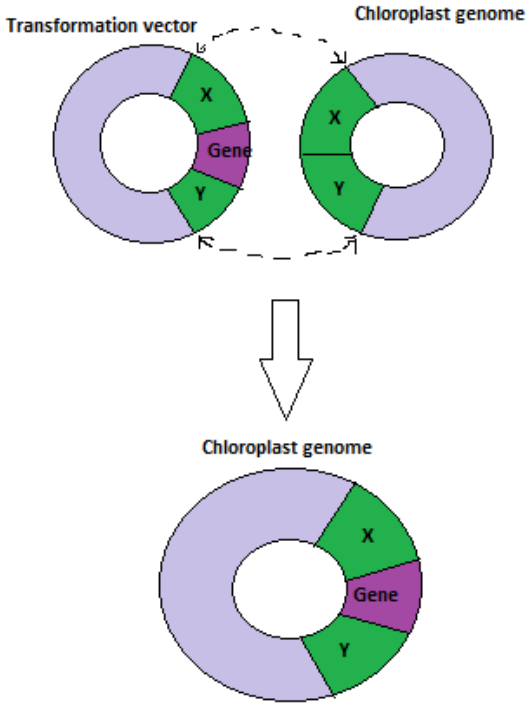

Figure 10: Homolog recombination. The figure gives an overview of the integration of a transgene into the circular chloroplast genome of *C. reinhardtii* by homolog recombination

1.4.3 How to express genes in *C. reinhardtii*

One of the most successful methods of transforming plants is by bombarding target cells with DNA-coated tungsten or gold particles. This way of transforming plants is both simple and effective. The kinetic energy of the particles is great enough to penetrate the cell wall, the plasma membrane and even the two membranes surrounding the chloroplast. The particles can of course also deliver multiple copies of the transgene into the chloroplast. A minor drawback of the method is the cost for the required special equipment and consumables, e.g. gold particles, and that the number of positive transformants can be quite low after a bombardment. But today this is the most efficient and effective method for chloroplast transformation in use (Potvin and Zhang, 2010).

1.4.4 Ectoine in metabolic engineering

Agrobacterium tumefaciens has been used in nuclear transformation in land plants. The *ectABC* cluster has been successfully integrated into tobacco and tomato plants, and the genes were transcribed using endogenous plant promoters. Accumulation of ectoine was detected in both plants, and they showed increased resistance towards salinity when compared with their wild-types. These experiments gave valuable insight into the mechanisms responsible for plant growth, salt tolerance and effectiveness of ectoine (Moghaieb et al., 2006) (Moghaieb et al., 2011). The problem with expression of transgenes in plastids is that some tissues of food plants and fruits don't contain chloroplasts but amyloplasts and chromoplasts. Scientists believe that the activity of plastid gene expression is lower in non-photosynthetic tissue, than in photosynthetic tissue, but today too little is known about the bottlenecks of protein expression in non–green plastids (Bock, 2007).

1.4.5 Codon optimization

All amino acids, except methionine and tryptophan, are encoded by more than one triplet codon. Such codons usually differ by one nucleotide in the third position, and are called synonymous. Synonymous codons are rarely used with equal frequencies in different organisms. Bias in codon usage is an essential feature of most genomes, both bacterial, archean and eukaryotic (Ermolaeva, 2001).

Optimizing the codons in the transgenes increases their expression efficiency by increasing their translation rate. It may also decrease their susceptibility to silencing. Scientists believe that for prokaryotic genomes and organelle genomes, e.g. in the chloroplast genome of *C. reinhardtii*, codon bias is the single most important determinant for successful protein expression. Therefore, optimisation of codons in transgenes is considered necessary for high level protein expression (Potvin and Zhang, 2010). For a graphical view of codon optimization, see appendix 2.

1.4.6 Previous work with the *ect* genes and *C. reinhardtii*

In previous attempts to produce ectoine in the chloroplast of *C. reinhardtii*, the bacterial *ectA* gene has been inserted into a suitable vector and transformed into the algae's chloroplast by

microprojectile bombardment. In a later experiment both the *ectA,* and the *ectB* genes were inserted together in the same vector, and transformed into the chloroplast of *C. reinhardtii*. In both cases it was found that both the *ectA* and *ectB* genes were expressed, but at a very low level. Neither ectoine accumulation in the cells or increased salt tolerance of the algae were detected. Codon optimising the *ect* genes has not been done before in *C. reinhardtii.* These projects have been carried out in cooperation with the Microbiology Department of the University of Bonn, Germany,

Aim of project

The main goals of this project were:

1. To create a transgenic *Chlamydomonas reinhardtii* cell line that contains the functional genes *OectA*, *ectB* and *OectC*.
2. Evaluate the expression of the *OectA*, *ectB* and *OectC* genes, and the effects of production of ectoine.

Sub-goals to achieve the main goals were:

1. To create a plasmid construct that works well for transformation of the chloroplast genome with all the three genes *ectA*, *ectB* and *ectC*. *ectA* and *ectC* will be codon optimized to fit the demands of *C. reinhardtii*. The constructs will also contain promoters and terminating regions for each gen, a selection marker (in this case the *atpB* gene), and a sequence that is homologous to chloroplast DNA for the stable homolog integration of the construct (in this case a BamHI-EcoRI insert). The flanking 5' and 3' untranslated regions (UTR) of each gene shall enable stabilization and translation of the transcript.

2. Transformation of *C. reinhardtii* with the constructed plasmid by microprojectile bombardment.

3. Selection of positive transformants, and analysis of them at the DNA, RNA, protein and product (ectoine) level.

2.0 Materials and Methods

2.1 Work with DNA

2.1.1 Quantification of DNA by Ultra violet light absorption

Procedure:

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

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

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

2.1.2 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.

2.1.3 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.

2.1.4 Purification of DNA fragments from agarose gel

*Specific DNA samples were isolated and purified by using the Illustra^{TM} purification kit from GE Healtcare.

Procedure:
-Cut out the DNA fragment of interest using UV-light at 350 nm (so the DNA is not damaged).
-Add a minimum of 300 µL Capture buffer, and use 10 µL per 10 mg after that. Mix by inversion.
-Use a heating block at 60^{o}C 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 g 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 g 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 000g in room temperature.
-Store DNA sample (flow through) at -20^{o}C, or use immediately.

2.1.5 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 -20^{o}C for minimum 30 minutes (can also freeze overnight).

-Centrifuge at 4^{o}C for 10 minutes at 13 000 g.

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

-Add 1.0 mL of 70% ethanol.

-Centrifuge at 4^{o}C for 5 minutes at 13 000 g.

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

2.1.6 Dephosphorylation of DNA (Method 1)

Procedure:

-Resuspend precipitated DNA in 90 µL dH_2O and add 10 µL of buffer.

-Add 1µL CIP enzyme, and mix with pipette.

-Putt the sample on a heating block at 37^{o}C for 1 hour.

-Inactivate CIP by heating at 75^{o}C for 10 minutes.

-Precipitate DNA as described in section 2.1.5.

2.1.7 Dephosphorylation of DNA (Method 2)

Procedure:

-Resuspend precipitated DNA in 90µL dH_2O and add 10 µL of buffer.

-Add 1µL CIP enzyme, and mix with pipette.

-Putt the sample on a heating block at 37^{o}C for 1 hour.

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

-Centrifuge at 13 000 g 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 g 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 g at 4^{o}C for 10 min.

-Discard supernatant, and add 1 mL of ethanol (70%) to the pellet. Mix by inversion.
-Centrifuge at 13000 g 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.

2.2 Work with Escherichia coli

2.2.1 Preparation of agar growth plates with ampicillin

Procedure for 1 L (approximately 40 plates):
-1 L of lysogeny broth (LB) with 1.5% agar 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.

2.2.2 Transformation of competent *E. coli* cells

Procedure:
-Melt an aliquot of frozen competent cells on ice, and add pure DNA (1 ng/µL) or 3 µL of a ligation reaction. Mix and leave tube on ice for 30 minutes.
-Incubate on a heating block at 42ºC 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 (described in section 2.2.3).

2.2.3 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 a petri dish (dilute the culture if to 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^{o}C for 16-20 hours (incubation is not done in the hood).
-Store at 4^{o}C or use immediately. (Can be stored in the cold for several weeks).

2.2.4 Culturing of *E. coli* agar plate colonies

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 37^{o}C on a rotating wheel.
-The next day, the culture can be used for plasmid isolation (Miniprep). (See section 2.2.6).

2.2.5 Storage of *E.coli* at -80^{o}C

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 -80^{o}C freezer.
*Cells are viable for several years.

2.2.6 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 (4^{o}C).
-Centrifuge at 13 000 g 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 g 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 g 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 g 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 g at 4ºC for 10 min.
-Discard supernatant, and add 1 mL of ethanol (70%) to the pellet. Mix by inversion.
-Centrifuge at 13 000 g 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.
- Use immediately, or store in freezer at 20ºC.

*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.

2.2.7 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 37ºC on a shaker.
-Centrifuge at 6000 g at 4ºC 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 g 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 g 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 g in room temperature.
-Transfer supernatant to Beckman OptiSeal tubes, and load tube into VTi 65.2 rotor.
-Centrifuge at 50 000 g 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). (See section 2.1.1.).
-Store in the freezer at 20^{o}C.

2.3 Cloning

2.3.1 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^oC 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^oC for minimum 3 hours.

-Use immediately or store at -20^oC.

2.3.2 Digestion with restriction enzymes

Procedure:

All restriction digestions were done with enzymes from NEB (New England Biolabs), following the protocols recommended by the supplier. The overall restriction mixture is a mix of Enzyme, DNA, dH_2O, buffer and if needed BSA.

2.3.3 Plasmids

The *ectABC* gene cluster from *P. stutzeri* was obtained on a PSB01 plasmid provided by the University of Bonn, Germany (Appendix 3). The pMU_kn+ plasmid were synthesized by Life Technologies™ GeneArt® service. The plasmid SK+_157_NdeI (the plasmid SK+_157 modified to have a NdeI site instead of an Eco47 site) is described in (Salvador et al., 2011). The final plasmid for transformation into the chloroplast of *C. reinhardtii*, the pCrc_32 plasmid is described in (Blowers et al., 1993). The SK+ 157_NdeI plasmid, the pCrc_32 plasmid and pMU-RQ plasmid contain an ampicillin resistance gene. Transformants containing these plasmids or derivatives were selected on petri dishes containing ampicillin.

The pMU_kn+ plasmid contained a kanamycin resistance gene. Transformants with this plasmid were selected on petri dishes containing kanamycin.

2.3.4 Codon optimization of *ectA* and *ectC*

To codon optimized sequences of *ectA* and *ectC* the online tool "Graphical codon usage analysis", and the function "each triplet position vs usage table" (http://gcua.schoedl.de/sequential_v2.html) was used (Fuhrmann et al., 2004). The online tool (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055.chloroplast&aa=1&style=N) was used to get the *C. reinhardtii* chloroplast codon usage table (Nakamura et al., 2000). The original *ectA* and *ectC* sequences used are from *Pseudomonas stutzeri* (Seip et al., 2011) see Appendix 2.

2.4 Work with *Chlamydomonas Reinhardtii*

2.4.1 *C. reinhardtii* strains

The non-photosynthetic *atpB* deletion mutant strain ac-uc-221 (CC373), and the wild type strain (CC125) of *C. 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, and the wild type and transformants are maintained in the high salt (HS) media. The mutants can not perform photosynthesis and will die when exposed to light in a medium not containing an organic carbon source.

2.4.2 Preparation of solid media for *C. 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.

2.4.3 Preparation of liquid medium for *C. 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.

2.4.4 Culturing the *C. reinhardtii* photosynthesis mutants for transformation

Procedure:

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

2.4.5 Harvesting and plating of the *C. reinhardtii* photosynthesis mutant for transformation

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

Procedure (6 plates):

-Centrifuge 500 mL of the *C. reinhardtii* mutant culture in two sterile centrifuge tubes at 5000 g 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 sterilized 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 *C. 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. Don not turn the plates upside-down.
-Store the plates in the dark for minimum 3 hours before microprojectile bombardment.

2.4.6 Microprojectile bombardment

*Transformation vector DNA is precipitated, and pasted on gold particles according to instructions of the manufacturer of the particle delivery system (Bio-Rad).

*The following steps are done in as little light as possible.

Procedure:
-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 containing photosynthesis mutants.
-The 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 the plates up-side down.

2.4.7 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 turned upside-down. They are kept under constant light.

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

2.4.8 Photosynthetic growth of *C. reinhardtii*

When growing *C. 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 24 hours, night and day.

2.4.9 Photosynthetic growth of *C. reinhardtii* with 2% CO_2

*Prior to DNA and RNA isolation *C. 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 *C. reinhardtii* 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 *C. 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.

2.4.10 Total DNA isolation from C. reinhardtii

Procedure:

-Centrifuge 40-80 mL (depending on how long the cells have grown, and hence their concentration) of a *C. reinhardtii* culture containing about 2 x 10^6 cells per mL for 5 minutes in room temperature at 5000 g.

-Discard supernatant and resuspend the pellet in 0.75 mL DNA extraction buffer (100 mM Tris pH 8.0, 50 mM Na_2-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 g.
-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 g.
-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 g. Discard supernatant, and add 1 mL of ice-cold ethanol (70%). Mix by inversion.
-Centrifuge for 2 minutes in room temperature at 13 000 g. 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 Na_2-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 g. Discard supernatant, and add 1 mL of ice-cold ethanol (70%). Mix by inversion.
-Centrifuge for 5 minutes in 4ºC at 13 000 g. Discard supernatant, and dry pellet in a vacuum centrifuge (or by incubation at room temperature).
-Resuspend DNA in 20 µL off sterile dH_2O. Measure DNA concentration by dot spot (see section 2.1.2), and store in freezer (-20ºC) or use immediately.

2.4.11 RNA isolation from *C. 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 *C. reinhardtii* culture containing approximately 2 million cells per mL for 5 minutes at 5000 g 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 Na_2-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 g 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 g 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 g 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 g 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 $OD_{260\ nm}$ (OD 1.0 = 40 µg RNA/mL). To the rest of the DNA solution add 10 µL Na-acetate (3M pH 5.2) and add 200 µL ice-cold ethanol (96 %). Mix and incubate at -20ºC for minimum 1 hour.
-Centrifuge for 10 minutes at 13 000 g in 4ºC. Discard supernatant and add 1 mL of ethanol (70 %), mix by inversion.
-Centrifuge for 10 minutes at 13 000 g 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.

2.5 Analytical methods

2.5.1 Preparation of radioactive probes (random primer labelling) for DNA and RNA blots

Synthesis of the radioactive probe was done with a DNA polymerase (Klenowe fragment). The polymerase is primed by random hexanucleotide primers. In the deoxynucleotide triphosphate (dNTP) mix the deoxycytidine triphosphate (dCTP) has the radioactive ^{32}phosphor isotope incorporated at its α-phosphate ((α-^{32}P)-dCTP), which gives a high energy β-particle emission when the isotope decays (half life 14.3 days).

*Work with high energy radioactive material should only be done by authorized personal, and with proper shielding and protective measures.

Procedure:

-Prepare a DNA template for the radioactive probe.

-In a 1.5 mL microfuge tube mix 10.5 µL sterile dH_2O, 2 µL template DNA (100 – 200 ng/µL).

-Denature in boiling water for at least 5 minutes, and add 5 µL cold labelling buffer, 2.5 µL BSA (4 mg/mL), 1.5 µL dNTP mixture (deoxyadenosine triphosphate, deoxythymidine triphosphate and deoxyguanosine triphosphate (1 mM of each, mixed 1:1:1)).

-Add 2.5 µL (25 microcurie (µCi)) (α-^{32}P)-dCTP, and mix with a pipette.

-Add 1 µL DNA polymerase I (Klenow fragment) (2 units/mL) (NEB).

-Spin briefly and incubate at room temperature for 3 hours.

-Store at -20^{o}C.

2.5.2 Hybridizing sample DNA and RNA with radioactive probes made by random primer labelling

Procedure:

-The hybridization buffer is stored at 37^{o}C.

-Wash blotting membrane in dH_2O.

-Put the membrane into a hybridization tube and add 1 mL of hybridization buffer. Incubate at 65^{o}C on a rotating wheel for 15 minutes.

-Discard the hybridization buffer and add 4 mL hybridization buffer.

-Add random primer labelled probe at a ratio of 1/2000 the amount of hybridization buffer (e.g. 10 mL hybridization buffer requires 5 µL probe).
-Incubate for ca. 24 hours at 65^{o}C on a rotating wheel.
-Dispose of the radioactive hybridization buffer properly, put wash buffer 1 and wash buffer 2 at 65^{o}C water bath.
-Wash membrane at 65^{o}C on a rotating wheel. Wash twice with wash buffer 1for 5 minutes, and then seven times for 5 minutes in wash buffer 2. Do a final wash for 20 minutes in wash buffer 2.
-Wrap membrane in plastic wrap, with the side with DNA having a smooth surface.
-Check radioactivity with a Geiger counter.
-Visualize the result of the hybridization by autoradiography.

2.5.3 Autoradiography

*The Autoradiography was done with the Kodak® BioMax® MS equipment and solutions.

Procedure:
-In a dark room, fix the blotting membrane to the audioradiography casing, and insert a x-ray film with intensifying screen. Close the casing.
-Expose film at -80^{o}C using a few hours, or overnight, depending on how fresh the radioactive probe is, and how much hybridization occurred.
-Develop the film in a dark room by dipping the film for 1 minute into developer solution, rinse in dH_2O, then dip the film in fixer solution for 1 minute.
-Rinse the film in plenty of water, and dry. Take photos with a digital camera.

2.5.4 Slot blot

Procedure:
-Add 3 µL NaOH (5 N) to a 1.5 µL microfuge tube.
-Add 500 ng sample DNA dissolved in dH_2O, and if necessary add sterile dH_2O to raise the volume to 50 µL.
-Denature by incubating at 65^{o}C for 45 minutes. Cool down to room temperature, and add 50 µL saline-sodium citrate buffer (SSC) (10 x).
-Setup the dot blot apparatus (PR 600 SlotBlot, Hoefer Sientific Instruments (USA) according to operation instructions.

-Soak blotting membrane for 5 minutes in dH_2O, then for 5 minutes in SSC (10 x).
-Load blotting membrane into dot blot apparatus, and pull 200 µL SSC (10x) through sample slot by suctioning with a vacuum aspirator.
-Turn off suction, and add 100 µL SSC (10x) in sample slot. Add denatured DNA sample and mix with a pipette in the slot. Turn on suction and wait until all liquid has been sucked through.
-Turn off suction, and wash slot by sucking through 200 µL SSC (10x).
-Take out the blotting membrane, put it into 2X SSC for 20 seconds, and then wrap it into plastic, and crosslink DNA to membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
-Hybridize the membrane with a radioactive probe and develop by autoradiography.

2.5.5 Southern blot

Procedure:
-Isolate genomic DNA (section 2.4.9).
-Digest in a microfuge tube 1.5 µg of genomic DNA in 20 µL with 30 units of enzyme for 4 hours.
-Ethanol precipitate the digested DNA.
-Resuspend DNA in 20 µL, including DNA gel loading buffer.
-Run reaction on an agarose gel.
-Check gel under UV-light, and take a photo with a digital camera.
-Transfer DNA to a nylon membrane according to protocol.
-Put membrane into plastic wrap, and crosslink DNA to membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
-Hybridize the membrane with a radioactive probe and develop by autoradiography.

2.5.6 Southern blot analysis

-Take a photo of the autoradiographically developed Southern blot results.
-Print out the picture and cut out (with a scissor) around the result of the transformed, and un transformed plastomes in each sample.
-Use the analytical-weight and measure the weight of the transformed an untransformed plastomes for each sample.

-Calculate the ratio between them to see how many plastomes are transformed. Together they represent 100% of the plastomes in the transformant.

2.5.7 Northern blot

* All equipment and solutions used have to be RNase-free.

Procedure:

-Sterilize an electrophoresis chamber, a try and a comb with 3% H_2O_2 overnight.

-Dissolve 0.78 g agarose in 37 mL DEPC-treated water (1.3% agarose gel). Cool down in water bath at 65^{o}C.

-Add 12 mL MOPS buffer (5x), and 11 mL formaldehyde (37%).

-Mix gently by swirling and pour into a gel tray. Put in comb.

-Prepare the samples by mixing for each sample in a microfuge tube:

2.5 µL DEPC – treated water

2 µL MOPS buffer (5x)

3.5 µL formaldehyde

3.5 µL ethidium bromide (100 µg/mL)

10 µL formamide

2 µL RNA sample (2 µg/mL)

-Incubate samples at 65^{o}C for 15 minutes.

-Put the gel into the electrophoresis chamber and add running buffer to cover gel (315 mL DEPC H_2O , 90 mL MOPS (5X), and 45 mL formaldehyde).

-Prerun for 5 minutes at 60 mA.

-Cool down samples, spin in centrifuge at max speed for 1 minute, and add 2 µL RNA gel loading buffer.

-Mix with a pipette and add 20 µL sample per well.

-Run the samples at 60 mA for 10 minutes in reverse direction, than in normal direction until the bromphenol blue band is at the bottom of the gel.

-Take a photo of the gel.

-Wash briefly in DEPC-treated water and transfer RNA to a nylon membrane following the protocol.

-Transfer for 6 hours. Put membrane into SSC (2x). Check gel under UV-light for complete transfer.

-Wrap membrane into plastic wrap, and crosslink DNA to membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
-Hybridize the membrane with a radioactive probe and develop by autoradiography.

2.5.8 Aligning agarose gel photo to autoradiographic films

Procedure:
-Take a photo of the agarose gel with ladder, or rRNA bands, making sure that both top and bottom of the gel is included for reference points.
-When continuing the blotting procedure, make sure that the bottom of the blotting membrane is aligned with the bottom of the agarose gel.
-Continue the blotting procedure until a film is developed.
-Mark the point on the film that was the bottom of the blotting membrane, and the point 8.5 cm above that (the agarose gel is 8.5 cm). This gives the top and bottom reference points. Photograph the film.
-On the computer align the bottom point of the gel to the bottom point of the film, and the same for the top point.

2.5.9 Salt tolerance experiment

50 mL solutions of high salt (HS) media with NaCl concentration 0%, 0.5% and 1% were made. 50 mL of transformant cultures, in the exponential phase, were poured into each salt solution. The mixtures were left for 24 hours (12 hour light/12 hours dark) before analysing the result using your eyes to see colour differences.

3.0 Results

3.1 Construction of the transformation plasmid

3.1.1 The *ectABC* genes

The goal of this project was to introduce the *ectA, ectB* and *ectC* genes from *P. stutzeri* into *C. reinhardtii* in order to create salt tolerant *Chlamydomonas* transformants. The *ectA* and *ectC* genes were codon optimised for expression in *C. reinhardtii* chloroplasts. These genes were designated *OectA* and *OectC*. The *ectB* gene was not optimised. In *P. stutzeri* the *ectABC* genes are expressed as an operon. In our plasmid construct each gene was linked to separate promoters and a transcription termination sequence. *OectA* was linked to the 5' region of the *rbcL* gene (coding for the rubisco large subunit) and the 3' region of the *psaB* gene (coding for subunit B of photosystem I). The *ectB* coding sequence was linked to the 5' region of the *psbD* (coding for a photosystem II subunit) and the 3' region of the *rbcL* gene. *OectC* was linked to the 5' region of the *atpH* gene (coding for an ATPase III subunit), and the 3' region of the *petA* gene (coding for a cytochrome f). All flanking regions were endogenous *Chlamydomonas* chloroplast sequences. Construction of the transformation vector containing the *ect* genes is described in the following paragraphs.

3.1.2 Cloning of *OectA*

The cloning strategy for the *OectA* construct is shown in **Figure 11.** Plasmid SK+_157_NdeI (Lunde, 2012) was digested with *Nde*I and *Xba*I, resulting in the removal of the ~ 2kb *GUS* gene from the SK+_157_NdeI vector. The *OectA* gene fragment of 582 bp ending in restriction sites *Nde*I and *Spe*I, was ligated into the *Nde*I/*Xba*I-digested SK+_157_NdeI vector creating the SK+_157_OectA plasmid. *Spe*I and *Xba*I ends are compatible (**Figure 12)**. After transformation into competent C2529 *E. coli* cells (NEB), plasmid DNA from transformants was isolated and analysed by digesting with *Nde*I and *Xba*I which should release the inserted *OectA* fragment of 582 bp from the constructed SK+_157_OectA plasmid (**Figure 13**). A positive transformant containing the *OectA* gene fragment was selected for maxiprep.

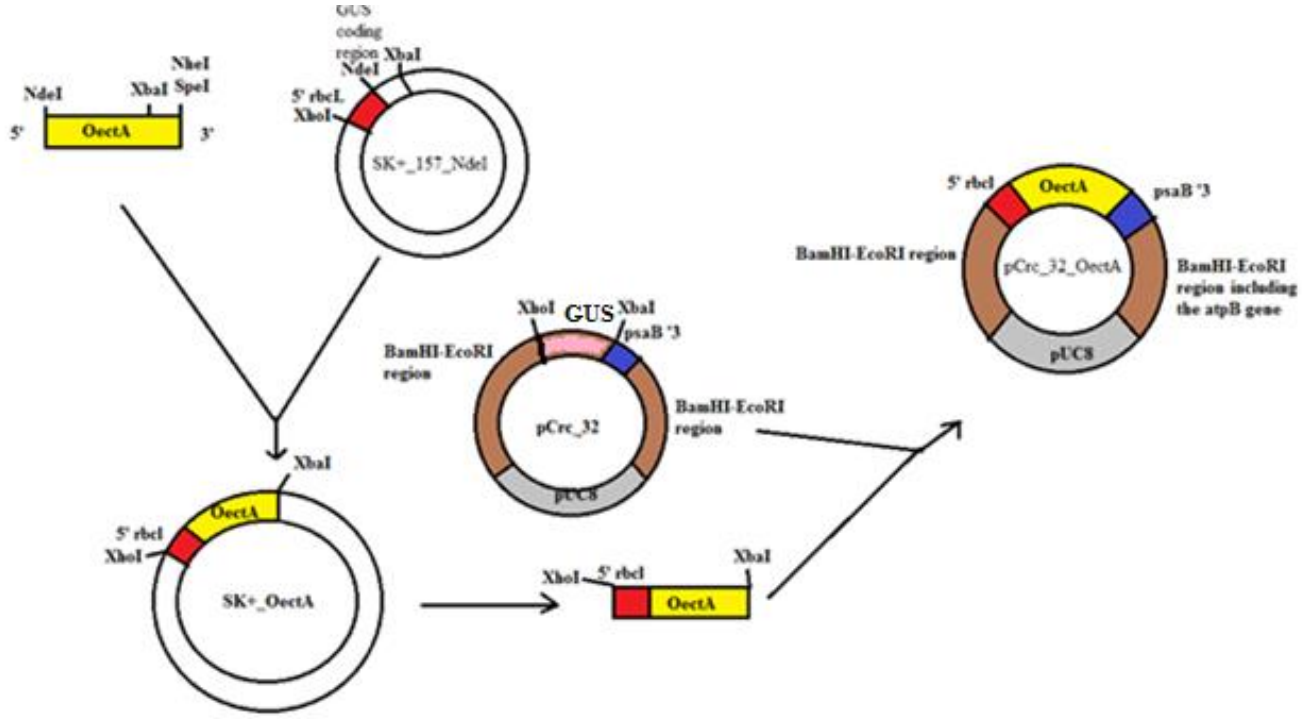

Figure 11: Cloning strategy of *OectA*. The *OectA* fragment was ligated into the *Nde*I and *Xba*I site of SK+_157_NdeI, next to the *rbcL* region creating the SK+_OectA plasmid. A fragment containing both the *rbcL* region and the *OectA* was released with *Xho*I and *Xba*I from this plasmid and ligated into the *Xba*I and *Xho*I site of the pCrc_32 plasmid, after removing a *GUS* gene ~ 2000 bp (pink), creating the pCrc_32_OectA plasmid.

SpeI	XbaI	NdeI
5' ACTAGT 3'	5' TCTAGA 3'	5' CATATG 3'
3' TGATCA 5'	3' AGATCT 5'	3' GTATAC 5'

Figure 12: Restriction sites for *Spe*I, *Xba*I and *Nde*I. The restriction sites of *Spe*I and *Xba*I are compatible with each other.

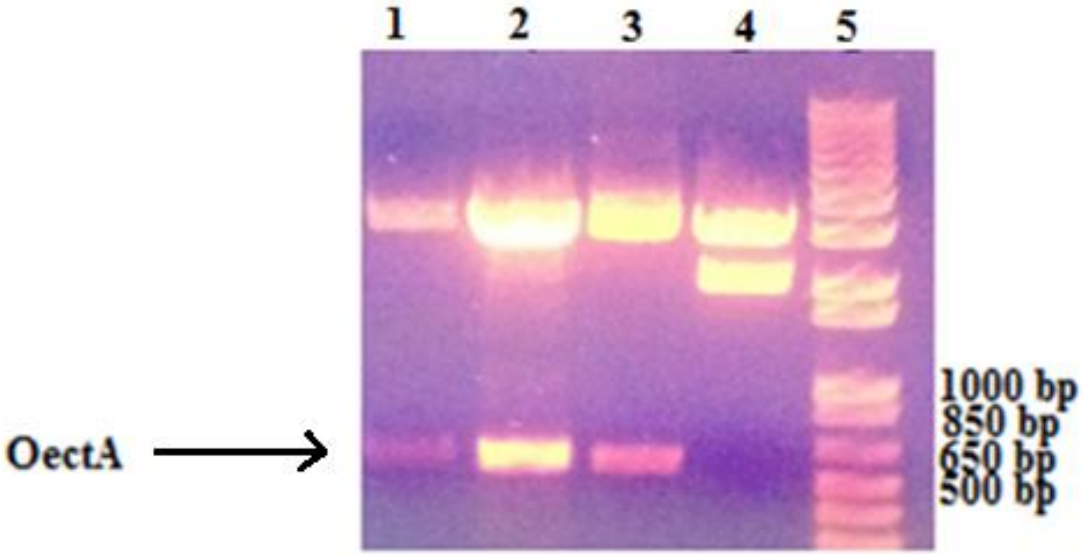

Figure 13: Analysis of miniprep DNA to identify transformants carrying the OectA gene construct. Isolated plasmid DNAs were digested with *Nde*I and *Xba*I. *OectA* (582 bp) was released from three transformants (lanes 1-3). Lane 4: The SK+_157_NdeI plasmid used as a control. Lane 5: 1 kb ladder (Appendix 5).

In order to clone the *OectA* construct into the *Chlamydomonas* chloroplast transformation vector, the SK+_OectA construct was digested with *Xho*I and *Xba*I and the resulting 743 bp fragment, containing the *OectA* construct, was cloned into *Xho*I/*Xba*I-digested plasmid pCrc_32. This released the ~2000 bp *GUS* gene in pCrc_32 with the *OectA* construct (*GUS* gene in pink in **Figure 11**). The pCrc_32_OectA plasmid was transformed into competent *E.coli* TB1 cells. Plasmid DNA from transformants was analysed by digesting with *Pst*1. (**Figure 14**). The *Pst*1 digestion would give a fragment of about 2100 bp if the ~2000 bp *GUS* gene was removed and replaced with the *OectA* 743 bp fragment .Plasmids from transformants still containing the *GUS* gene will give a fragment of around 3400 bp whereas plasmids from *OectA*-positive transformants should release a *Pst*1-fragment of ~2100 bp. Of four analysed transformants, three contained inserts of the anticipated size. One of the transformants containing the *OectA* gene fragment was selected for maxiprep.

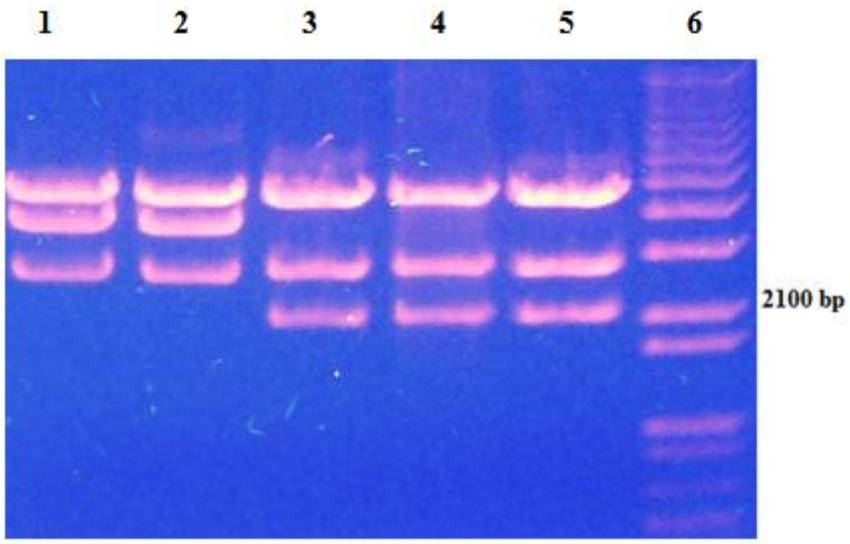

Figure 14: **Restriction enzyme digest of the pCrc_32_OectA plasmid with Pst1.** The pCrc_32_OectA plasmid was digested with *Pst*1 to analyse the transformants for insert size. Lane 1: Vector control, pCrc_32 plasmid digested with *Pst*1. Lanes 2-5 transformants. Lane 6: 1 kb ladder. Lanes 3-5 show plasmids with the expected insert.

3.1.3 Cloning of *OectC*

Next, the OectC construct, containing the atpH and petA flanking regions (see 3.1.1) was inserted into the pCrc_32_OectA plasmid. The cloning strategy is shown in **Figure 15**. The pCrc_32_OectA plasmid was digested with restriction enzyme *Nhe*I. This restriction enzyme has only one restriction site in the vector. To avoid relegation of the linearized pCrc_32_OectA plasmid during ligation, the plasmid was dephosphorylated with calf intestine phosphatase (CIP) after digestion with *Nhe*I.

To isolate the 700 bp *OectC* fragment (including the 5'*atpH* and 3'*petA* regions), the pMU_kn+ plasmid was digested with *Nhe*I and the *OectC* construct purified from an agarose gel. The purified fragment was ligated into the dephosphorylated linearized pCrc_32_OectA plasmid. *OectC* fragment has *Nhe*I restriction sites in both ends of the fragment and can therefore ligate with the vector in two different orientations. After transformation into competent *E. coli* TB1 cells, plasmid DNA was isolated from transformants, digested with *Nhe*I and analysed by gel electrophoresis (**Figure 17**). Only one of the eight transformants appeared to contain the *OectC* fragment (lane 6). This transformant contained the pCrc_32_OectA_OectC plasmid (Appendix 4). The other transformants gave two large bands

presumably linearized and supercoiled plasmid without insert. The transformant containing the *OectC* gene fragment from lane 6 was selected for maxiprep to obtain enough DNA for further cloning work.

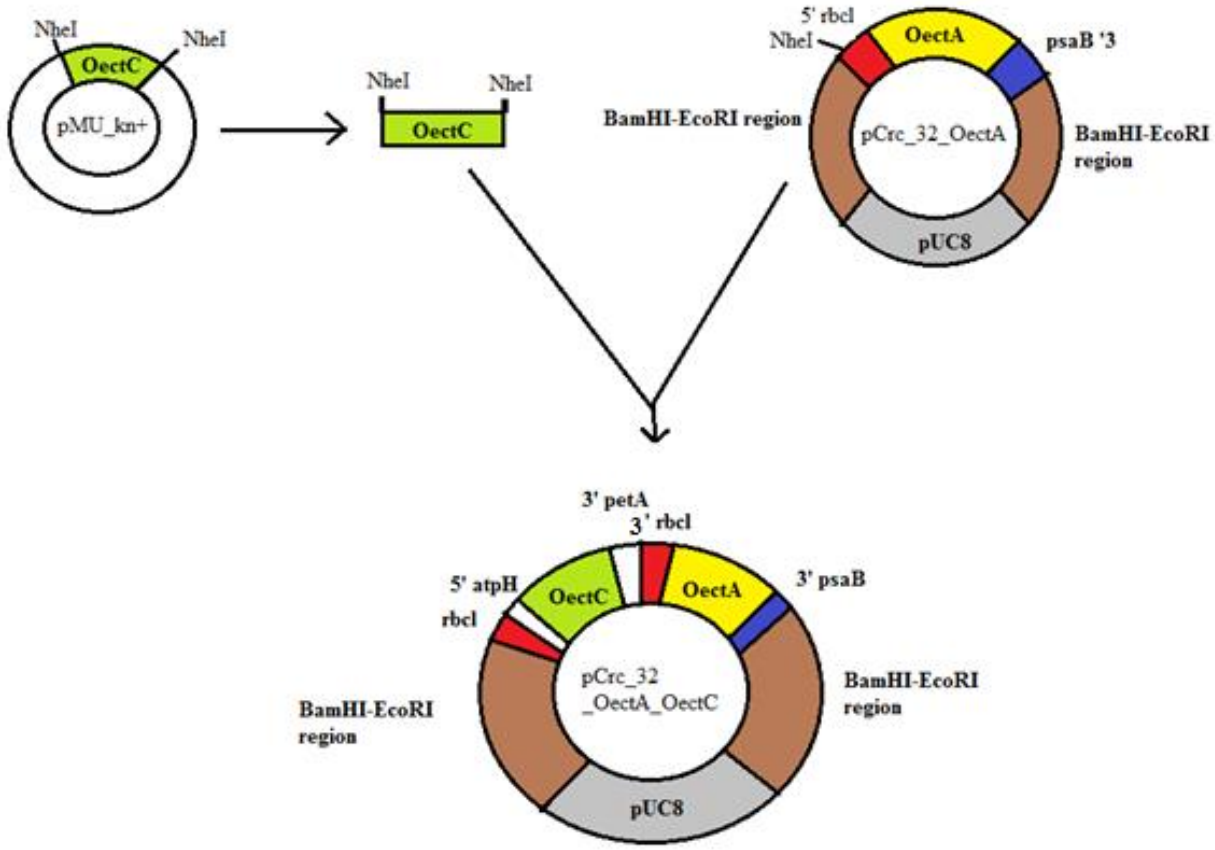

Figure 15: Cloning strategy of *OectC*. The *OectC* construct was released from the pMU_kn+ vector by digestion with NheI, and isolated from an agarose gel. The vector pCrc_32_OectA was digested with the restriction enzyme NheI (**Figure 16**), and ligated to the *OectC* fragment. The resulting pCrc_32_OectA_OectC plasmid is approximately 9800 bp.

NheI
5'..GCTAGC..'3
3'..CGATCG..'5

Figure 16: Restriction site of the restriction enzyme *Nhe*I.

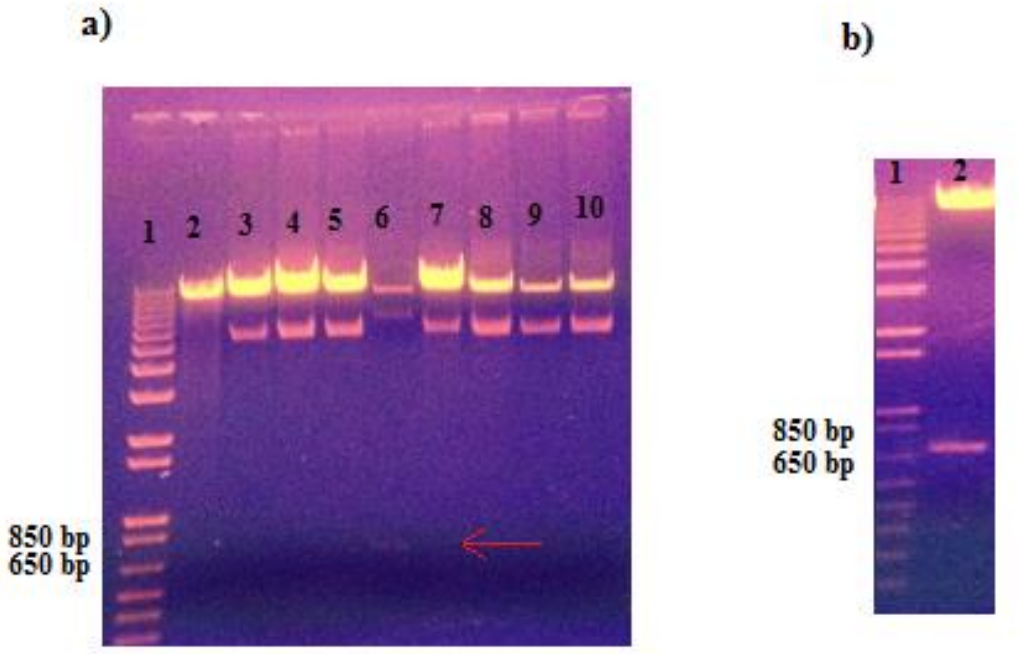

Figure 17: NheI digestion of miniprep DNA to identify transformants that harbour the *OectA*/*OectC* constructs. a) Lane 1: 1 kb ladder. Lane 2: Linearized pCrc_32_OectA plasmid (control). Lanes: 3-10 plasmid DNA from transformants. Red arrow points to a weak band of ~700 bp. **b)** Repetition of digestion from transformant from lane 6 using larger amounts of DNA to confirm the presence of a 700 bp fragment.

3.1.4 Attempted cloning of *ectB*

The cloning strategy for insertion of the *ectB* construct into the pCrc_32_OectA_OectC plasmid is presented in (**Figure 18**). The pCrc_32_OectA_OectC plasmid was digested with XhoI and dephosphorylated with CIP to avoid relegation during the ligation reaction. To obtain the *ectB* gene fragment (including the 5' *psbD* promoter region), the pMU_RQ plasmid was transformed into competent *E. coli* TB1 cells. pMU_RQ plasmid was isolated from a transformant and digested with *Sal*1. The fragment presumably containing *ectB* was purified by agarose gel electrophoresis and subsequent excision from the gel. The purified fragment was ligated into the dephosphorylated linearized pCrc_32_OectA_OectC plasmid. *Sal*1 and *Xho*I are compatible restriction sites (**Figure 19**). The insert can be ligated into the vector in both orientations. The ligation mixture was cloned into competent *E. coli* TB1 cells. Plasmid DNA was purified from transformants, digested with *Sal*1 and analysed by agarose gel

electrophoresis. Several cloning experiments were carried out. In all cases only religated pCrc_32_OectA_OectC plasmids were obtained from the transformants.

Since C. reinhardtii already contains the enzyme L-2,4-diaminobutyrate transaminase as part of its amino acid metabolism, performing the same reaction as the enzyme encoded by *ectB* (**Figure 7**), the lack of *ectB* in the transformation vector was not considered to be critical for ectoine synthesis in *C. reinhardtii*.

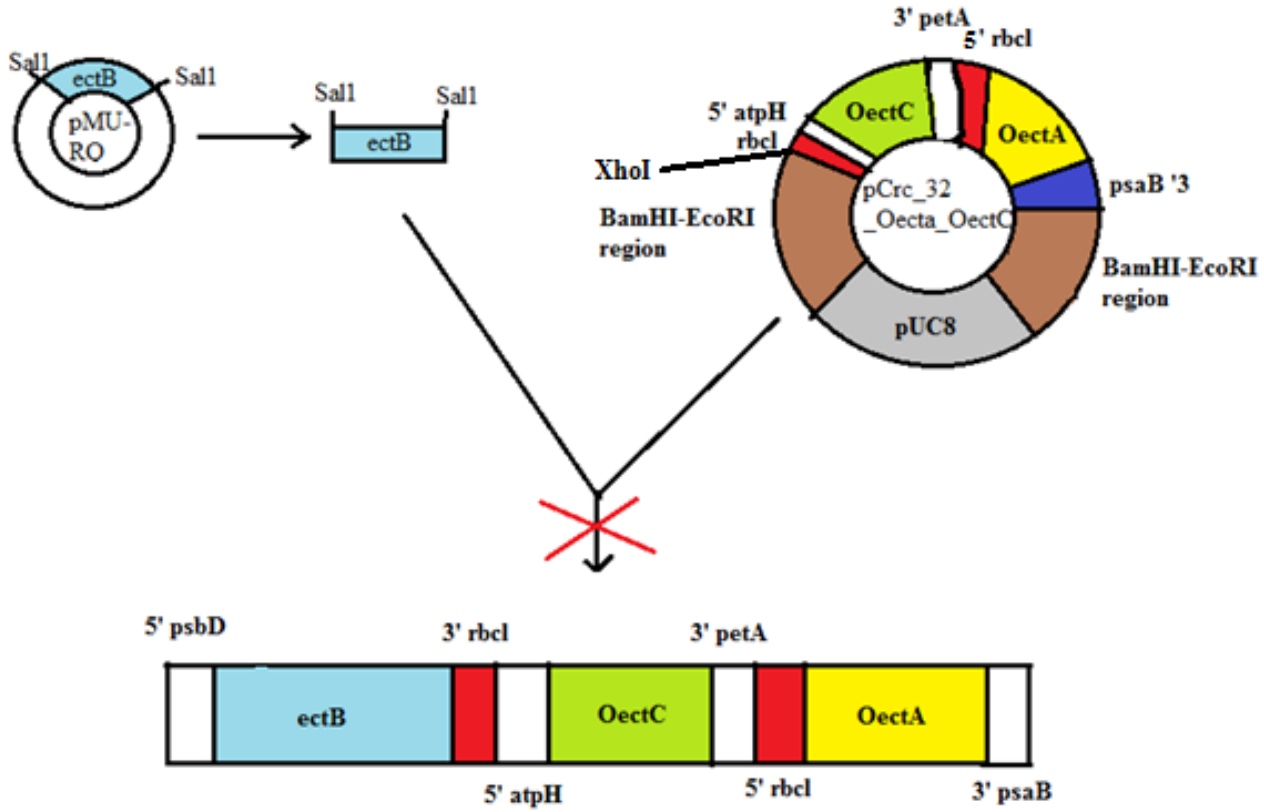

Figure 18: Attempted cloning of *ectB*. *ectB* was released from the pMU-RQ plasmid by *Sal*1 and isolated from an agarose gel. The pCrc_32_OectA_OectC vector was digested with *Xho*I and dephosphorylated with CIP. Unfortunately, all attempts to ligate *ectB* with the pCrc_32_OectA_OectC vector failed. The red cross marks the step that would not work. The desired final insert is shown at the bottom of the figure.

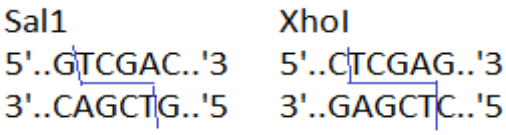

Figure 19: Restriction sites for the restriction enzymes *Sal*1 and *Xho*I. The sticky ends made by the two restriction enzymes are compatible with each other.

Because we encountered difficulties in cloning *ectB*, we wanted to investigate if the *ectB* contained an additional unknown *Sal*1 site, which could jeopardise the cloning. To test this we digested the original PSB01 plasmid containing the *ectABC* operon from *P. stutzeri* with *Sal*1 (**Figure 20**). From the known DNA sequence (Appendix 3) this plasmid should only contain one *Sal*1 site in the *ectA* gene. The appearance of a separate 1100 bp fragment indicated the presence of an additional *Sal*1 restriction site 1100 bp from the known *Sal*1 site in *ectA*. If the additional *Sal*1 site is downstream of the *ectA* gene it will be present in the *ectB* gene. To determine this, we digested the PSB01 plasmid with *Sal*1 and *Bgl*II, and *Sal*1 and *Xho*I (**Figure 21**).

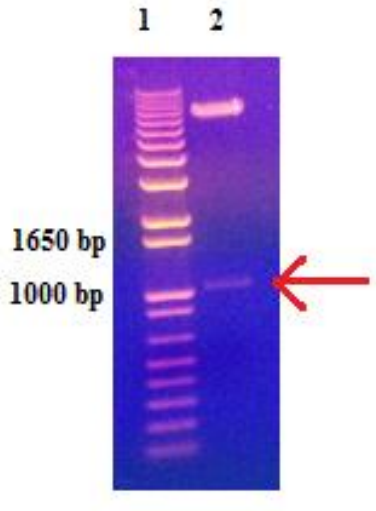

Figure 20: PSB01 plasmid containing the *ectABC* gene cluster digested with *Sal*1. Lane 1: 1 kb ladder. Lane 2: Sal1 digest of PSB01 plasmid. The arrow indicates a fragment of about 1100 bp.

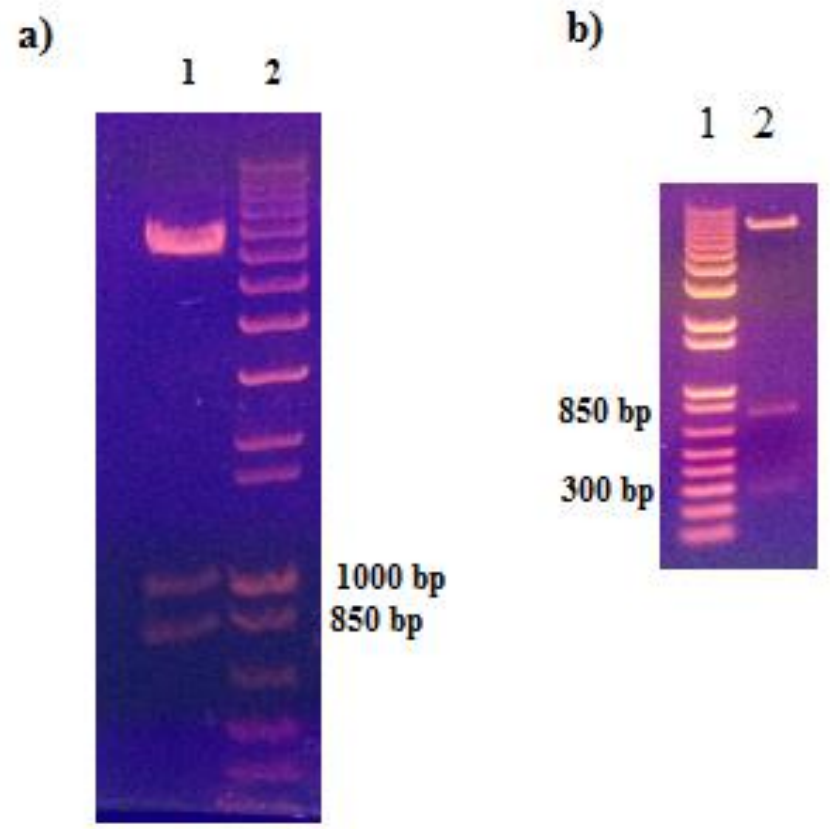

Figure 21: PSB01 plasmid digested with a) *Sal*1 and *Bgl*II **b)** *Sal*1 and *Xba*I. In a) Lane 1: plasmid digest. Lane 2: 1 kb ladder. In b) Lane 1: 1 kb ladder. Lane 2: plasmid digest.

From the known *Sal*1 site in *ectA* it is approximately 1800 bp to the *Bgl*II site in *ectC*. We obtained two fragments of about 1000 bp and 800 bp when digesting with *Sal*1 and *Bgl*II. This is consistent with the presence of an additional *Sal*1 site in the *ectB* gene. When digesting with *Sal*1 and *Xba*I we obtained two fragments of about 270 bp and 750 bp. From the known sequence we expected only a ~270 bp fragment. This confirms the *Sal*1 site in *ectB*.

3.2 Transformation and selection of *OectA* and *OectC* transformants in *C. reinhardtii*

3.2.1 Microprojectile bombardment and selection for transformants

The pCrc_32_OectA_OectC plasmid contains the full-length *atpB* gene which can complement the mutated *atpB* gene in the *C. reinhardtii* strain (ac-uc-2-21) used for transformation. Uptake of the *atpB* gene restores photosynthetic abilities and facilitates selection of transformants. The *atpB* gene is part of a 5.3 kb sequence from the *Chlamydomonas* chloroplast genome that directs integration of transformed DNA into the chloroplast DNA by homologous recombination.

Gold particles of 0.6 µm were coated by precipitation with the pCrc_32_OectA_OectC plasmid. The plasmid was introduced into to a *C. reinhardtii atpB* deletion mutant by biolistic transformation using a particle bombardment device. After bombardment, the transformed cells grew for 2 weeks on high salt (HS) plates under constant light for phototrophic selection. Only transformants containing the full length *atpB* gene should be able to grow. Individual colonies were then transferred to high salt (HS) liquid media and left to grow for 4 days with constant light. Transformants 1, 2, 4, 8, 11 and 25 were selected for their rapid growth. These transformants were grown with additional 2 % CO_2 for 4 more days with 12 hours light/12 hours dark cycles.

3.2.2 DNA slot blot

DNA slot blotting was carried out to analyse the six transformants for the presence of the *OectC* gene. DNA was isolated from transformants 1, 2, 4, 8, 11, and 25, and 500 ng DNA was transferred to a membrane in a slot blot apparatus. A ^{32}P-radiolabled probe was prepared from the *OectC* fragment, and hybridised to the genomic DNA on the membrane. Hybridisation signals were visualized using autoradiography (**Figure 22**). The slot blot showed that the *OectC* gen had been integrated into the chloroplast genome of *C. reinhardtii* in transformants 1, 2, 4, 11, and 25. Even though we see a weak signal in transformant 8, this is so faint that it most likely consist of background noise. As there was not enough time for another round of screening, we decided to proceed with analysis of transformants 2 and 25, although their slot blot signals were also not strong compared to the controls.

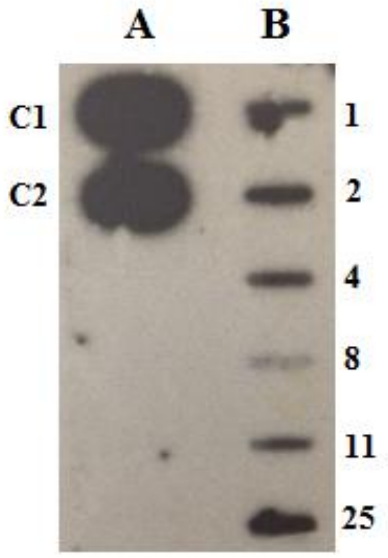

Figure 22: DNA hybridisation signals from transformants after DNA slot blotting using an *OectC* probe. **A**: controls C1, and C2 consisting of the pCrc_32_OectA_OectC vector. **B**: DNA from *C. reinhardtii* transformants 1, 2, 4, 8, 11, and 25.

3.2.3 Southern blot

A Southern blot was carried out to investigate how many copies of the plastid genome of transformant 2 and 25 harbored the *OectA* and *OectC* genes (**Figure 23**). DNA was taken from the same samples used to make the slot blot and digested with *EcoR*I and *Kpn*I. This would give us a fragment of about 5000 bp containing the *OectA*, the *OectC*, and the *atpB* genes.

Prior to the Southern analysis it was discovered that two copies of the *OectC* gene were present in the transformation vector. The pCrc_32_OectA_OectC plasmid was digested with *Swa*I and *Xho*I which should release a fragment of 770 bp containing the *OectC* gene. Instead, a fragment of approximately 1450 bp was observed (**Figure 24**). This indicates that the *OectC* gene has been incorporated in duplicate.

No *OectC* gene could be detected in transformant 2 by Southern analysis **(Figure 23).** In transformant 25, only about 5% of the plastomes were estimated by to carry the *OectAC* construct, by comparing the band intensities of the ~3300 and the ~5700 bands.

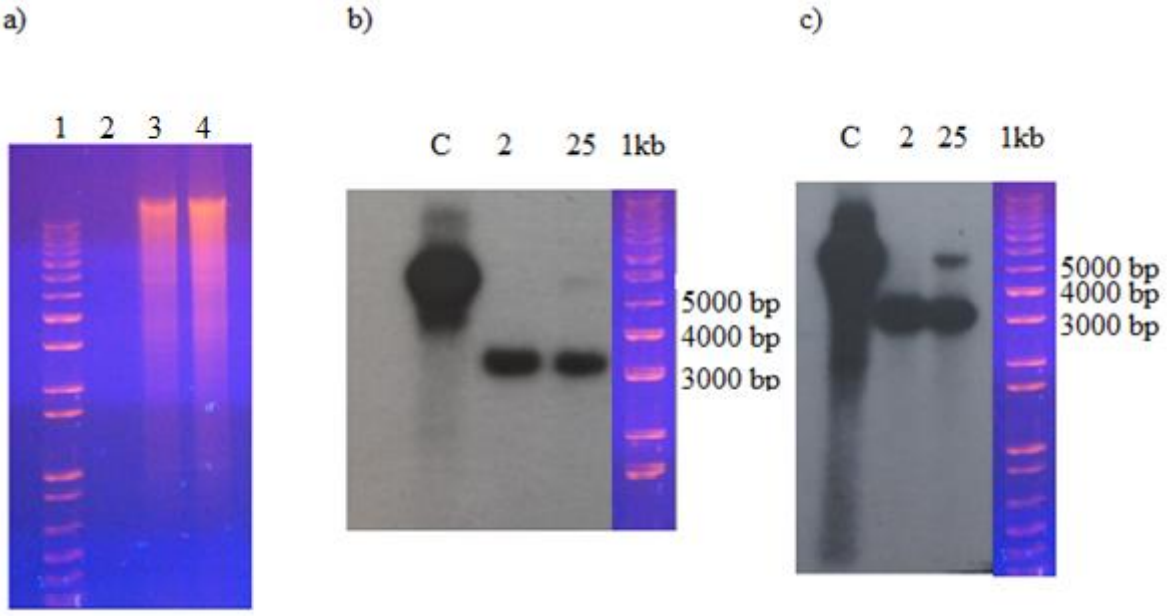

Figure 23: Gel electrophoresis before, and autoradiography after a Southern blot from transformants 2 and 25, hybridized with an *atpB* probe. a) Gel electrophoresis. Lane 1: 1Kb ladder. Lane 2: pCrc_32_OectA_OectC plasmid (control). Lane 3: Transformant 2. Lane 4: Transformant 25. **b)** x-ray film exposed for 3 hours. C: Wild type *C. reinhardtii* (control). 2: Transformant 2. 25: Transformant 25. **c)** x-ray film exposed for 24 hours. C: Wild type *C. reinhardtii* (control). 2: Transformant 2. 25: Transformant 25. Only transformant 25 seems to have plastomes containing the insert.

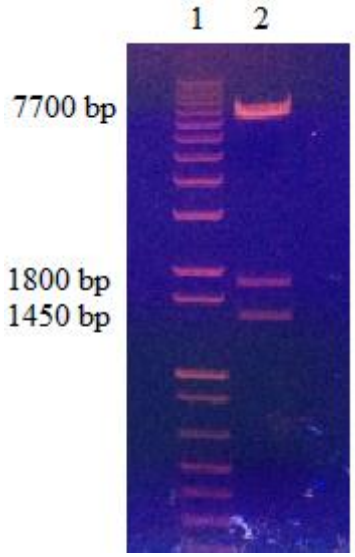

Figure 24: Restriction enzyme digest of the pCrc_32_OectA_OectC plasmid with *Swa*I and *Xho*I. Lane 1: 1 kb ladder. Lane 2: Restriction digest.

3.2.4 Northern blot

A northern blot was carried out to investigate if *OectC* was transcribed into mRNA in transformants 2 and 25. The transformed algae cultures were grown in 12 hours night/12 hours day cycles. Total RNA was isolated after 1 hour growth in light, and subjected to agarose gel electrophoresis. The RNA was subsequently transferred to a membrane by northern blotting and hybridized to a radiolabelled probe for the *OectC* gene. The signals were visualized by autoradiography (**Figure 25**). The autoradiography was examined by visual inspection revealing faint bands in the transformants, not present in the control. The apparent size of the bands was around 700 bp, which is the expected size of the *OectC* transcript, indicating a very low level transcription of the *OectC* gene.

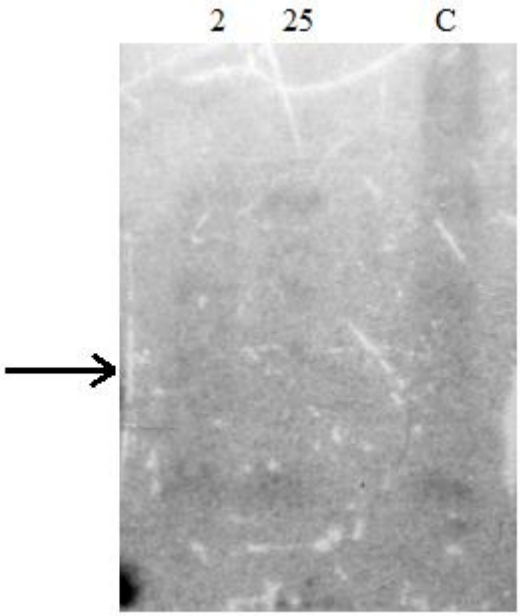

Figure 25: Autoradiography after northern blot from transformants 2 and 25 hybridized to the *OectC* probe. Lanes marked 2 and 25 indicate RNA from transformants 2 and 25, respectively. Lane C: control, RNA from untransformed *C. reinhardtii*. The arrows indicates faint bands approximately 700 bp in the transformants.

3.2.5 Salt tolerance experiment

Wild type *C reinhardtii* is sensitive to NaCl. The algae can survive in HS medium supplemented with 0.5% NaCl, but will not grow. To investigate if the transformed algae showed enhanced salt tolerance, we grew transformant 2 in HS medium containing 1% NaCl.

Transformant 25 was grown in HS medium containing 0.5% NaCl, and 1% NaCl. The results of the growth experiment after 24 hours of growth are shown in **Figure 26.** The colours of the transformant cultures in HS media with added NaCl were significantly less intense compared to the controls. The transformant in 0.5% NaCl solution shows somewhat more colour than those in the 1% NaCl solutions. *C. reinhardtii* transformant nr 25 in 0.5% NaCl appears to survive, but not grow. These results indicate that the transformants have not obtained higher salt tolerance.

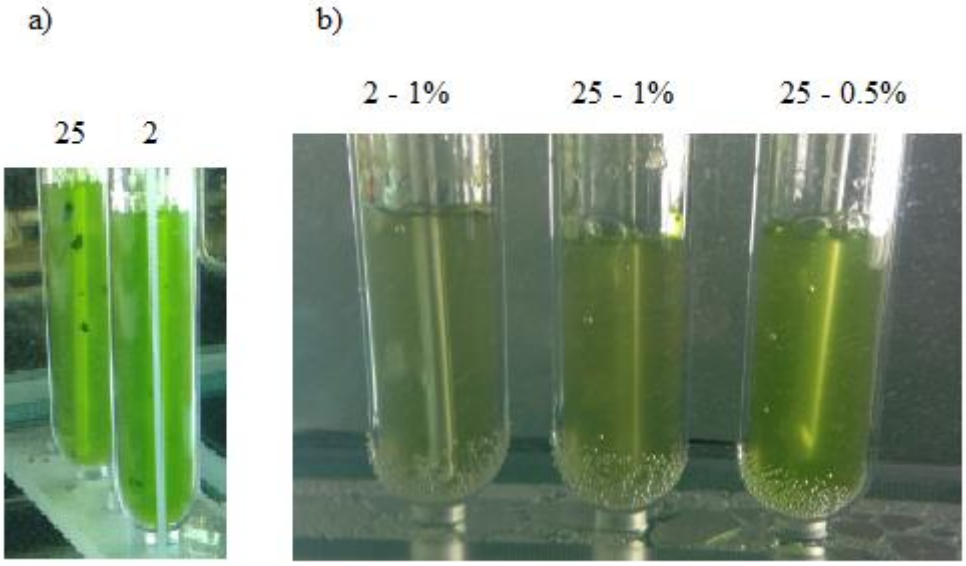

Figure 26: Behaviour of *C. reinhardtii* transformants in NaCl solutions. a) Transformants 2 and 25 grown in HS medium without added NaCl (controls). **b)** Transformant 2 in HS medium with 1% NaCl (left), transformant 25 in HS medium with 1% NaCl (middle), and transformant 25 in HS medium with 0.5% NaCl (right).

4.0 Discussion

4.1 Cloning of the *ect* genes

The main goal of the project was to synthesize ectoine in the chloroplast of *C. reinhardtii*. Therefore we wanted to introduce the *ectA, ectB* and *ectC* genes from *P. stutzeri* into the chloroplast genome of *C. reinhardtii* and determine salt tolerance in ectoine-producing transformants. In *P. stutzeri* the *ectABC* genes are organized as an operon. Because operons have been found difficult to express in *C. reinhardtii* (Drapier et al., 1998) the genes were inserted into the chloroplast genome as individual constructs, each gene containing different promoter and terminating sequences.

The plasmid used for transformation was constructed by using the backbone of the pCrc_32 plasmid from which the *GUS* gene has been removed. The pCrc_32 plasmid was created by Blowers et al. (1993), based on the same construct design as in Blowers et al. (1989). This plasmid has previously been shown to be suitable for chloroplast transformation in *C. reinhardtii*. It contains the *atpB* gene which can be used for selection of transformants when introduced into *atpB*-deficient mutants of *C. reinhardtii* thereby restoring photosynthesis. The plasmid also contains sequences homologous to endogenous sequences in *C. reinhardtii* chloroplast DNA (the BamHI-EcoRI regions, see **Figure 11**) necessary for homologous recombination.

Previous research has shown that it is much more likely to get protein expression of transgenes in transgenic microalgae if the introduced genes are codon optimised (Potvin and Zhang, 2010). The *ectA* gene was previously optimised for expression in *C. reinhardtii*, and designated the *OectA* gene. We codon-optimised the *ectC* gene, and designated it *OectC*. In our construct we wanted primarily to use these codon optimised genes. This implied that we could not use already existing constructs containing non-optimised genes (e.g. *ectA/B* constructs) but had to make new constructs.

Cloning of *OectA* into the transformation vector was straight forward. Several transformants containing the *OectA* gene were obtained in the first cloning experiment. The subsequent cloning of *OectC* into the pCrc_32_OectA plasmid proved much more difficult. Both the *OectC* fragment and the pCrc_32_OectA vector were digested with *Nhe*I only. This creates insert and vector fragments with *Nhe*I compatible sequences at both ends. To avoid religation

the vector was dephosphorylated. However, several attempts to clone *OectC* were unsuccessful despite several different cloning strategies. Finally, we tried to inactivate CIP after dephosphorylation of the vector by phenol/chloroform/isoamylalcohol (25:24:1) extraction instead of the recommended heat inactivation. This seemed to have the desired effect. One possible explanation for this could be that when CIP is inactivated by heat at 75^{o}C, the temperature is so high that dsDNA denatures to ssDNA. Upon rapid cooling of the solution, ssDNA may not have time to reanneal. If the DNA remains single stranded it would not work in the subsequent ligation reaction. Using phenol/chloroform/isoamylalcohol inactivation of CIP, we were able to clone the *OectC* fragment into the pCrc_32_OectA vector, creating the pCrc_32_OectA_OectC plasmid.

The *ectB* gene was not codon optimised due to limited resources. This gene is comparatively larger (1278 bp) than *ectA* (582 bp) and *ectC* (401 bp). Cloning of *ectB* also proved difficult. Also here, the insert and vector were digested with one enzyme, *Sal*I, creating fragments with *Sal*I compatible ends. We did several transformation experiments, varying the same parameters as in the cloning of *OectC*. However, despite all variations in cloning experiments, we were unable to obtain any clones containing the *ectB* gene. Control digests of the original PSB01 plasmid containing the *ectABC* gene cluster confirmed the presence of an additional *Sal*I site in *ectB*. When examining the *ectB* sequence in the *ectABC* gene cluster provided, we found a sequence, 5'-GTCGAT-3', closely resembling a *Sal*I site 5'-GTCGAC-3'. Upon resequencing of the *ectB* gene, it became evident that this in fact was a *Sal*I site, explaining the fragment patterns obtained by agarose gel electrophoresis after digesting with *Sal*I, *Bgl*II and *Xho*I combinations.

Digesting the pMU_RQ plasmid with *Sal*I released a fragment of apparent molecular weight of 1600 bp as expected for the *ectB* sequence with promoter and terminating regions. As the pMU-RQ vector contains *Sal*I sites, it was possible that the 1600 bp fragment isolated from the gel was partially from the *ectB* fragment and partially from the pMU_RQ vector. However, as previously stated, none of the transformants contained any inserted fragments, but consisted only of religated vector.

Expression of *ectB* may not be necessary for ectoine synthesis in the *Chlamydomonas* chloroplast because an L- 2,4-diaminobutyrate transaminase should be present as part of arginine and proline metabolism in *C. reinhardtii*. Therefore we might naturally have enough substrate for the L-2,4-diaminobutyrate acetyltransferase (encoded by *ectA*) to continue the

synthesis from L-2,4-diamionobutyrate to N_2-acetyl-L-2,4-diaminobutyrate and further to ectoine.

4.2 Analyses of chloroplast transformants

The results of the slot blot suggested that five out of six analysed *C. reinhardtii* transformants contained the *OectC* gene (**Figure 22**). However, compared to the control, signals from the transformant DNAs were weak. Comparing our signals with signals on slot blots performed by previous master students the signals appeared weak (personal communication). This indicates that a low percentage of plastomes in the *C. reinhardtii* chloroplast have incorporated the *OectC* gene. To determine the percentage of plastomes that had incorporated the *OectC* a Southern analysis was made. During the search for restriction enzymes suitable for Southern analysis it was discovered that two copies of the *ectC* gene were present in the transformation vector (**Figure 24**). The orientation of the two *ectC* genes in the vector still needs to be determined. It is not known how a duplicate insertion of *OectC* will affect transcription.

The Southern blot analysis confirmed that both the *OectA* and the *OectC* genes were incorporated in about 5% of plastomes of transformant 25, and our suspicion that the percentage of plastomes containing *OectA* and *OectC* is low was confirmed (**Figure 23**). No plastomes containing the *OectA* or *OectC* were detected in transformant 2. The reason for this might be that too few plastomes have incorporated the *OectA* and *OectC* genes, so that the signal is too weak to be detected by Southern blotting. This seems the most likely explanation considering both the slot blot and the northern blot results. Another possibility is that we loaded a higher DNA concentration of transformant 2 compared to the other samples in the slot blot, so that what we actually thought was a positive transformant was background noise (unspecific hybridization to genomic DNA).

4.3 Transcript analysis

Expression levels of foreign genes in microalgae, in addition to generally being low, are inconsistent and difficult to predict. A significant part of this variation in expression levels arises from inconsistences in the number of transgene copies integrated within a particular

genome (Potvin and Zhang, 2010). Northern blot analysis of RNA isolated from transformants 2 and 25 indicated very low levels of *ectC* transcripts in both transformants. This fits our expectations for transformant 25 given the results in the slot blot and Southern blot. For transformant 2, the result appears inconsistent with the results of the Southern blot, but consistent with the result from the slot blot. The reason for low levels of expression may be low integration of the construct into plastomes (see section 4.2). Levels of chloroplast mRNAs may also be influenced by light. For example, the mRNA transcribed from the 5' *rbcL* UTR and promoter region upstream of the *OectA* gene is sensitive to light degradation (Salvador et al., 1993). As the RNA used in the Northern analysis was isolated in the light, stability might be a factor for levels of the *OectC* transcripts found. Transcript levels of *OectA* or the endogenous *ectB* homologue are not known but, if ectoine is produced, it is accumulated at a level which is so low that it does not significantly influence the salt tolerance of the transformed *C. reinhardtii* cells, as shown by the growth experiment (**Figure 26**).

It is in most cases desirable to design expression systems with high levels of expression. Accumulation of chloroplast gene transcripts can be impacted in a variety of ways during synthesis. For example during transcription, mRNA accumulation, translation or protein turnover. Plastid protein accumulation has been shown to depend mostly on the rate of translation, and the accumulation of proteins is not rate limiting in *C. reinhardtii*. Also it has been shown that mRNA production and protein production are well correlated (Rosa. León et al., 2008). According to Barnes et al. (2005) the 3' UTR region used in chimeric constructs seems to have little or no effect on the mRNA accumulation, but the 5' UTR and promoter region seems to be crucial. **Figure 27** shows different *C. reinhardtii* chloroplast promoters used as 5' UTR and promoter for a transgene (in this case *GFP*), and their correlation with mRNA and protein accumulation.

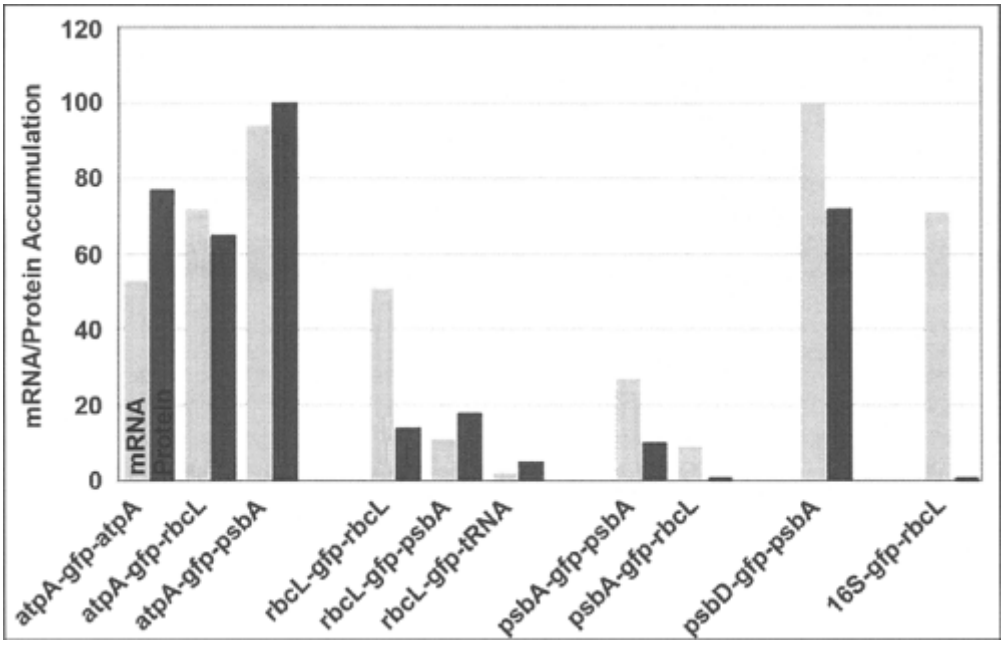

Figure 27: Analysis of mRNA/protein accumulation of *GFP* using different 5' UTR and promoter regions. mRNA and protein levels were normalised to 16S and Rubico levels, respectively. Grey columns: mRNA accumulation. Black columns: Protein accumulation. Figure from *Transgenic microalga as green cell factories* (Rosa. León et al., 2008).

Promoters from the *atpA*, and the *psbD* genes seem to work best in expression of the GFP gene in *Chlamydomonas*. Both mRNA and protein accumulation using these two promoters are high. For the *OectC* we had chosen *atpH* as 5'UTR and promoter. *atpH* is not shown here, but it can be assumed that the *atpH* 5' region directs transcription of transgenes as frequently as the 5' region of *atpA*. Both *atpH* and *atpA* genes encode different subunits of the ATPsynthase and should be expressed at the same rate. However, it has been documented that at least in *E. coli*, *atpA* mRNA is translated at least three times more efficiently than the *atpH* mRNA (Rex et al., 1994). Whether this is also the case for *C. reinhardtii* is not known. The promoter activity might also change when placed in front of a transgene. The *rbcL* promoter as we see here leads to both quite low mRNA and protein accumulation. We also know that *OectA* transcripts which contain the *rbcL* 5' UTR have a half-life of 5 hours in the dark, and only 20 minutes in the light (Singh et al., 2001). It could be that exchanging the 5' *rbcL* region with a more suitable 5' UTR and promoter region would give a higher transcription rate of the *OectA* gene and better transcript stability.

4.4 Conclusion

Understanding ectoine expression and its effect on salt tolerance can be of importance both in agriculture and in industry. We were able to transform *C. reinhardtii* with a plasmid construct containing the *OectA* and the *OectC* gene. *OectC* was expressed in transformants at a very low level. The expression of *OectA* and endogenous *ectB* in transformants remains to be determined. Ectoine did probably not accumulate in the pCrc_32_OectA_OectC transformants to any significant level since we did not observe any increased salt tolerance. Further work is needed to improve expression of the required genes of the ectoine biosynthetic pathway to achieve appreciable ectoine production.

4.5 Further Work

To complete this study a number of analyses needs to be done which, due to strict time limits for this master project, could not be addressed.
First of all, a new round of transformant screening should be done in order to find a transformant that is homoplasmic for the *OectAC* genes. Also, one should analyse the transformants for presence of the *OectA* and *OectC* proteins. Antibodies or tagging of the proteins could be used for detection and quantification.

Since codon optimization is regarded as the single most important determinant for successful protein expression (Potvin and Zhang, 2010), one should also construct a codon optimised *ectB* gene. This *OectB* gene should then be introduced into the pCrc_32_OectA_OectC plasmid and transformed into the chloroplast of *C. reinhardtii*. The whole bacterial pathway of ectoine synthesis would then be present in *Chlamydomonas* with codon optimised genes. This would increase the probability of ectoine production and, perhaps, induce salt tolerance in the algae. The rate of production and level of ectoine could be analysed by HPLC or mass spectrometry.

Even with all the genes present ectoine production could be so low that salt tolerance might not easily be detected. In this case one could investigate the effect of using other promoters, e.g. the *atpA* promoter presented in **Figure 27**.

A simpler cloning strategy would be to design the complete insert structure *in silico*, and order it as one large DNA fragment from a commercial DNA synthesis company. Different

varieties of the inserts containing different promoter regions could be used to compare the importance of the promoter regions for expression. Approximately the same amount of plastomes with insert must then be present in the transformants to make such a comparison.

When doing transformation experiments with *C. reinhardtii* one often obtains a large number of transformants. It could be interesting to analyse these phenotypically for salt tolerance early in the investigation process to identify the most promising transformants.

Appendix 1 - Solutions and recipes

Used in work with *E. coli*:

TEG (Tris-EDTA-Glucose) (1L)

25 mM Tris-HCl, pH 8.0, 10mM Na_2-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.
Kanamycin stock: In water 10 mg/mL. Use between 1-5 mL/L.

The antibiotic stock solutions have to be sterilized by filtration through a 0.22 µm filter and 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 dH_2O to 1L. To make 1x, dilute in dH_2O.

Agarose gel loading buffer

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

1% agarose (60 mL)

0.6 g agarose is added to 60 mL of 1 x TAE buffer. Solution is covered with plastic foil, and a microwave oven is used to make it boil. All the agarose most dissolve. Cool down to about 45^{o}C with running water before pouring it into a tray. The agar solidifies at approximately 40^{o}C.

Media for grooving 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).

Used in work with *C. reinhardtii*:

DNA extraction buffer

(100 mM Tris pH 8.0, 50 mM Na_2-EDTA, 0.5 M NaCl, 10 mM β-mercaptoeethanol).

Used for blotting:

20X SSC saline-sodium citrate

NaCl (3M), trisodium citrate (300 mM, pH 7.0 with HCl).

Labelling buffer

The following solutions most be made:

1. Mix 625 µL Tris (1M pH 8.0), 62.5 µL $MgCl_2$ (1M), 8.7 µL β-mercaptoethanol (14.4 M), and 303.8 µL sterile dH2O in a 1.5 mL microfuge tube. This gives a final solution of Tris-HCl (0.625 M pH 8.0), $MgCl_2$ (62.5 mM), β-mercaptoethanol (125 mM).
2. 2.5M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
3. Random hexanucleotide primers ($pd(N)_6$): 50 units/mL in TE (10 mM Tris, 1mM Na_2-EDTA, pH 7.5).

The final labelling buffer is a mix of 475 µL solution 1, 500 µL solution 2, and 25 µL of solution 3.

Used for hybridization:

Hybridization buffer (100 mL)

50 mL Na-phosphate buffer (1 M pH 7.2), 200 µL EDTA (0.5 M pH 8.9), 1g BSA, 7g SDS is mixed together, and dH_2O is added to 100 mL. Dissolve at room temperature (takes a long time).

Na-phosphate buffer (1 M pH 7.2)

134g Na_2HPO_4 x $7H_2O$ (or 89g Na_2HPO_4 x $2H_2O$, or 71g Na_2HPO_4) and 4 mL H_3PO_4 (85 %) is mixed, and dH_2O is filled to 1 L.

Wash buffer nr 1. (1L)

Mix 40 mL Na-phosphate buffer (1M pH 7.2), 2 mL EDTA (0.5 M pH 8.9), 5g BSA, 50 g SDS, and add dH_2O to 1 L.

Wash buffer nr 2. (4L)

Mix 160 mL Na-phosphate buffer (1M pH 7.2), 8 mL EDTA (0.5 M pH 8.9), 40 g SDS, and add dH_2O to 4 L.

Southern blot:

DNA gel loading buffer

4% sucrose and 0.25 % bromphenol blue.

Northern blot:

RNA gel loading buffer

Mix 0.5 mL glycerol (100 %), 4 µL Na_2-EDTA (250 mM pH 8.0), 2.5 mg Bromphenolblue, 2.5 mg Xylene cyanol FF, and add sterile dH_2O to 1 mL. Treat with DEPC before use.

MOPS buffer (5x)

Mix $MgSO_4$ (10 mM), MOPS (0.5 M), NaCl (2.5 M). Adjust pH to 7.5 with NaOH. Filter to sterilize and store in the dark.

Appendix 2 – Graphical codon usage analysis

The bar graphs below (**Figure 28** and **Figure 29**) show the difference between optimised and non-optimised codon usage of the *ectA* and *ectC* genes from *P. stutzeri* in the chloroplast of *C. reinhardtii.* To make the graphs we used the online tool "Graphical codon usage analysis", and the function "each triplet position vs usage table" (http://gcua.schoedl.de/sequential_v2.html) (Fuhrmann et al., 2004). We used the online tool (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055.chloroplast&aa=1&style=N) to get the *C. reinhardtii* chloroplast codon usage table (**Table 1**). We can also see the nuclear *P. stutzeri* codon usage for comparison in **Table 2** (Nakamura et al., 2000). In **Figure 28** and **Figure 29** each bar represent a codon and the bar value (relative adaptiveness) represents the average occurrence of the codon in *C. reinhardtii*. Red bars show codons with less than 10 % relative adaptiveness, grey bars show codons with less than 20 % relative adaptiveness, and the black bars show codons whit more than 20 % relative adaptiveness.

Table 1: Codon usage in the chloroplast genome of *C. reinhardtii*. The numbers from left to right after each codon triplet and one letter amino acid are: fraction for amino acid, frequency per thousand triplets, and number of instances in genome.

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

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

UUU	F	0.66	33.4	(894)	UCU	S	0.25	17.0	(455)	UAU	Y	0.71	24.6	(657)	UGU	C	0.84	7.6	(203)
UUC	F	0.34	17.1	(456)	UCC	S	0.04	2.8	(74)	UAC	Y	0.29	10.0	(266)	UGC	C	0.16	1.5	(39)
UUA	L	0.72	77.7	(2078)	UCA	S	0.33	22.0	(588)	UAA	*	0.84	2.9	(78)	UGA	*	0.03	0.1	(3)
UUG	L	0.04	4.3	(114)	UCG	S	0.06	4.0	(107)	UAG	*	0.13	0.4	(12)	UGG	W	1.00	13.5	(361)
CUU	L	0.13	14.3	(383)	CCU	P	0.35	15.5	(414)	CAU	H	0.53	10.1	(270)	CGU	R	0.70	32.4	(866)
CUC	L	0.01	1.0	(28)	CCC	P	0.08	3.4	(90)	CAC	H	0.47	8.8	(235)	CGC	R	0.09	4.1	(110)
CUA	L	0.06	6.4	(170)	CCA	P	0.53	23.6	(630)	CAA	Q	0.90	38.4	(1026)	CGA	R	0.07	3.4	(90)
CUG	L	0.03	3.7	(99)	CCG	P	0.05	2.4	(63)	CAG	Q	0.10	4.1	(110)	CGG	R	0.01	0.5	(14)
AUU	I	0.77	51.4	(1374)	ACU	T	0.37	24.4	(651)	AAU	N	0.70	42.1	(1126)	AGU	S	0.24	16.0	(428)
AUC	I	0.12	8.2	(219)	ACC	T	0.08	5.1	(135)	AAC	N	0.30	17.7	(472)	AGC	S	0.08	5.4	(144)
AUA	I	0.10	6.9	(184)	ACA	T	0.49	32.4	(865)	AAA	K	0.92	69.1	(1847)	AGA	R	0.11	5.3	(143)
AUG	M	1.00	22.3	(596)	ACG	T	0.06	3.9	(103)	AAG	K	0.08	6.2	(167)	AGG	R	0.02	0.9	(23)
GUU	V	0.46	29.3	(783)	GCU	A	0.53	34.0	(908)	GAU	D	0.72	25.3	(676)	GGU	G	0.70	44.0	(1177)
GUC	V	0.04	2.5	(68)	GCC	A	0.09	5.9	(159)	GAC	D	0.28	9.8	(263)	GGC	G	0.10	6.4	(172)
GUA	V	0.41	26.0	(696)	GCA	A	0.32	20.7	(554)	GAA	E	0.88	41.1	(1098)	GGA	G	0.14	8.6	(229)
GUG	V	0.09	5.6	(149)	GCG	A	0.05	3.3	(88)	GAG	E	0.12	5.7	(152)	GGG	G	0.06	3.7	(99)

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

Table 2: Codon usage in the nuclear genome of *P. stutzeri*. The numbers from left to right after each codon triplet and one letter amino acid are: fraction for amino acid, frequency per thousand triplets, and number of instances in genome.

Pseudomonas stutzeri **[gbbct]: 250 CDS's (76592 codons)**

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

```
UUU F 0.22  7.7 (   592)  UCU S 0.05  3.0 (   233)  UAU Y 0.32  8.3 (   632)  UGU C 0.20  2.5 (   190)
UUC F 0.78 27.2 (  2082)  UCC S 0.19 10.9 (   836)  UAC Y 0.68 17.3 (  1322)  UGC C 0.80 10.1 (   775)
UUA L 0.02  2.0 (   155)  UCA S 0.06  3.5 (   267)  UAA * 0.20  0.7 (    50)  UGA * 0.68  2.2 (   169)
UUG L 0.10 11.2 (   857)  UCG S 0.23 13.2 (  1013)  UAG * 0.12  0.4 (    31)  UGG W 1.00 14.5 (  1114)

CUU L 0.06  6.9 (   532)  CCU P 0.10  4.7 (   362)  CAU H 0.37  9.5 (   727)  CGU R 0.16 10.8 (   824)
CUC L 0.19 21.2 (  1620)  CCC P 0.26 12.7 (   974)  CAC H 0.63 15.8 (  1213)  CGC R 0.56 37.9 (  2906)
CUA L 0.04  4.7 (   361)  CCA P 0.12  5.7 (   434)  CAA Q 0.21  8.5 (   648)  CGA R 0.06  4.1 (   317)
CUG L 0.60 68.0 (  5211)  CCG P 0.52 25.3 (  1941)  CAG Q 0.79 32.2 (  2467)  CGG R 0.16 10.7 (   816)

AUU I 0.20 10.3 (   788)  ACU T 0.10  4.9 (   372)  AAU N 0.25  7.5 (   574)  AGU S 0.07  4.1 (   312)
AUC I 0.74 37.7 (  2889)  ACC T 0.59 28.3 (  2166)  AAC N 0.75 21.9 (  1678)  AGC S 0.38 21.7 (  1665)
AUA I 0.06  3.1 (   241)  ACA T 0.09  4.2 (   320)  AAA K 0.26  9.4 (   723)  AGA R 0.03  1.9 (   147)
AUG M 1.00 25.6 (  1962)  ACG T 0.22 10.8 (   824)  AAG K 0.74 26.5 (  2026)  AGG R 0.04  2.5 (   194)

GUU V 0.09  6.4 (   494)  GCU A 0.09 10.0 (   764)  GAU D 0.35 18.5 (  1416)  GGU G 0.17 13.4 (  1029)
GUC V 0.35 23.8 (  1820)  GCC A 0.50 52.9 (  4053)  GAC D 0.65 34.4 (  2634)  GGC G 0.64 49.6 (  3796)
GUA V 0.09  6.4 (   488)  GCA A 0.12 12.5 (   954)  GAA E 0.43 26.6 (  2039)  GGA G 0.07  5.6 (   427)
GUG V 0.46 31.6 (  2417)  GCG A 0.29 30.3 (  2317)  GAG E 0.57 34.9 (  2674)  GGG G 0.12  9.4 (   718)
```

Coding GC 61.64% 1st letter GC 64.49% 2nd letter GC 43.38% 3rd letter GC 77.05%

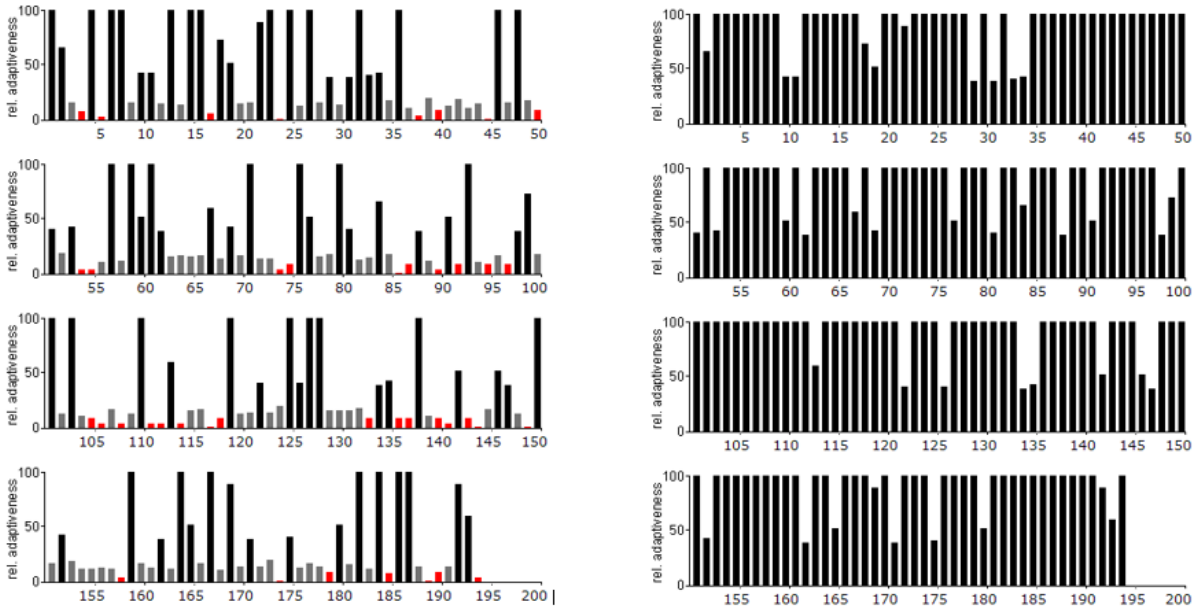

Figure 28: Codon usage *ectA* vs. *OectA*. The left bar graph shows codon usage of *ectA* from *P. stutzeri* in *C. reinhardtii* chloroplast. The right bar graph shows the usage *OectA* in *C. reinhardtii* chloroplast.

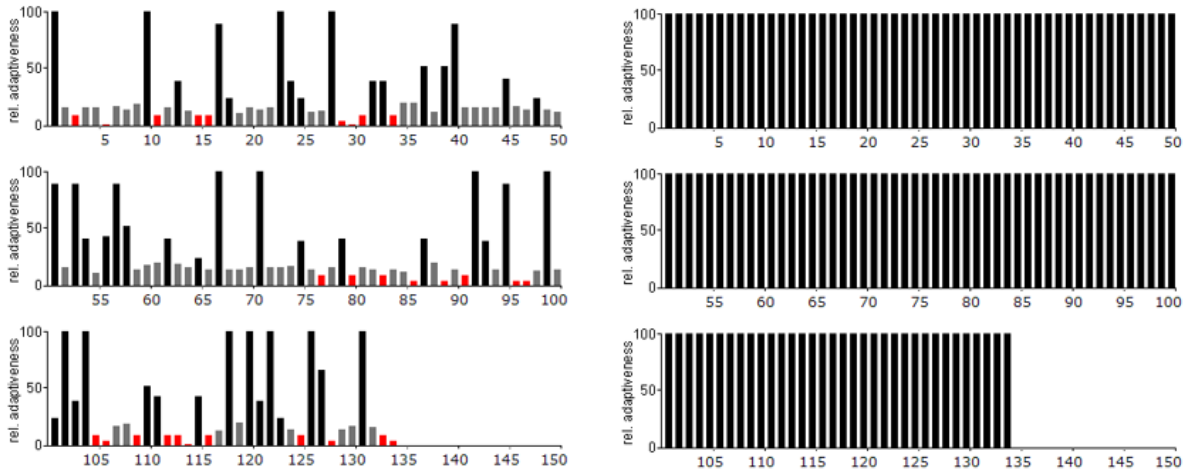

Figure 29: Codon usage *ectC* vs. *OectC*. The left bar graph shows codon usage in *ectC* from *P. stutzeri* in *C. reinhardtii* chloroplast. The right bar graph show codon usage of *OectC* in *C. reinhardtii* chloroplast.

Appendix 3 - The sequence of the *ectABC* gene cluster in *P. stutzeri*

Colour codes:

Yellow: *ectA* (overlapping area with *ectB* in bold letters)

Grey: *ectB*

Green: *ectC*

Red: *ectD*

Dark grey: *ask* (Regulatory gene)

NlaI

TTAAGAGCG**GGAGCC**GGGAAAACTGCGGAATATATGGGCGTCACACTAGCGCCATATAT**T** BspE

CCGGACTCGATCACAGAAGTCTTACGAATATCGAATCGCCCAGCGCGGCCTTATCCGCCC

AACCCCTTCGTTTGTCCCAGACGCCCCGCGCAAGCTACCAATCCGCCGCGCCAAGCACCG

CCACAGCCCTAGCCCGGCGCGGCTTTCAGCCAGGCAGACCGTCAGTCAGAGAACTTCTGA

CGCGCTCGGCGCTCGAACTTCCAGCCTGAATTCAAATTGCATTTCGGTGACAG**CTAAG**TT DdeI

GCCCGGCAAGCTGACCACCGCAATACACAGAAACATTCTGCGCGCCAGCATAGTTATCAT

GCGGGTTTCAGCGGCATATACAGCAACGCAAACTTCCCATATTCCGTTACATGGGTTGAG NdeI

AAACTTGTGCG**CAT****ATG**CCTACCCTAAAAAG**GAATTC**AATCAACAACCCCAAAGGCATTG EcoRI

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ATCAGCTGGTGGCGCGCTGCCAGCCCCTCGATACCAATTCGGTCTACTGCAACCTGCTGC

AGTGTTCCGATTTCGCTGACACCGCCATCGCCGCAGAGAACGCCCAAGGCGAGCTGGTCG

GTTTCATCTCGGGTTACCGCCCCCCTTCGCGGCCGGACACGCTGTTCGTCTGGCAGGTCG

CC**GTCGAC**AGTTCGATGCGCGGTCAGGGGCTGGCCCTGCGCATGCTGCTGGCACTGACCG SalI

CCCGGGTCGCTCGCGAGTACGGCGTGCGTTACATGGAAACCACCATCTCGCCGGACAACG

GGGCGTCACAGGCGCTGTTCAAGCGGGCCTTCGACCGCCTCGATGCCAACTGCACGACGC

GCACGCTGTTTGCCCGCGACACGCATTTCGCCGGTCAGCACGAGGACGAGGTGCTCTACC

GCGCCGGCCCGTTCACCGTTTCCCA**TCTAGA**AGAA**GAGCTC**AAGGAGCACGC**ATGA**AAAC XbaI SacI

TTTTGAACTGAATGAATCCAGG**GCTAGC**A**ACTAGT**GCCGTTCCTTCCCCGTGGTCTTCAA NheI SpeI

GCAGGCCCAGGGCGCCGAACTGGTCA**CTCAG**GACGGCAAGCGCTACATCGACTTCCTCGC DdeI

TGGTGCCGGCACGCTCAACTACGGGCACAACCACCCGGTGCTCAAGCAGGCGCTG**CTCGA** XhoI

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TTTCCTCGAAACCTTCAACCGGCTGATC**CTCGAG**CCGCGCGGCATGGGCGACTACCGCAT XhoI

GCAGTTCACCGGCCCGACCGGCACCAACGCGGTCGAGGCGGCGATGAAGCTGGCGCGCAA

GGTCACCGGGCGCAACAACATCATCAGTTTCACCAACGGCTTCCACGGCTGCAGCATTGG

CGCGCTGGCCGCCACCGGCAACCAGCATCACCGCGGCGGCTCCGGCATCGGCCTCACCGA

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GAAGCTCTGCCGCAAGCACGAGATGCTGCTGATCGTCGATGACATCCAGGCCGGCTGCGG

CCGCACCGGGACTTTCTTCAGCTTCGAAGAGATGGGCATCCAGCCGGATATCGTCACGCT EcoRV

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GGACCAGTGGAAGCCCGGCGAACACAACGGCACCTTCCGCGGCAACAACCATGCATTCGT

CACGGCGGCCGCGGCGGTCGAGCACTTCTGGCAGAACGACGCGTTCGCCAACAGCGTGAA

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GGCACTTTCCATCCTCGACAAGGCCTTTGCCGCCGTGATGAGCGAGCAGACCGAGAACCA HindIII

AGCTTCCTGAGGTATCCGCAATGATCGTCAGAACCCTCGCCGAGTGCGAAAAGACCGACC DdeI

GCAAGGTCCACAGCCAGACCGGCACCTGGGACAGCACGCGCATGCTGCTCAAGGACGACA

AGGTGGGATTCTCCTTCCACATCACCACCATCTACGCCGGCAGCGAGACGCACATCCACT

ACCAGAACCACTTCGAGTCGGTGTACTGCATCAGCGGCAATGGCGAGATCGAAACCATCG

CCGACGGCAAGATCTACAAGATCGAGCCGGGCACGCTGTACGTGCTGGAGAAGCATGACG BglII

AGCACCTGCTGCGCGGTGGCAGCGAAGACATGAAGCTGGCCTGCGTCTTCAACCCGCCGC

TCAACGGGCGCGAAGTGCATGACGAAAGCGGCGTCTATCCTCTGGAGGCCGAAACCGTCT

GATACCGGTTTAACCGGGGCGGCCACCGCGCCGCCCTGCCATTACAAGAAAGGAGGTAAG AgeI

CGTGAACCCTATGCAAGCCGACCTGTATCCCTCGCGCCAGGAAGACCAGCCCAGCTGGCA

GGAACGCCTGGATCCGGTCGTCTACCGCAGCGACCTGGAGAATGCGCCGATCGCGGCAGA BamHI

GCTGGTCGAACGCTTCGAACGCGACGGCTACCTGGTCATCCCCAATCTGTTCAGCGCCGA

CGAAGTCGCGCTGTTTCGCGCCGAACTCGAGCGCATGCGCCAGGACCCGGCCGTCGCCGG XhoI

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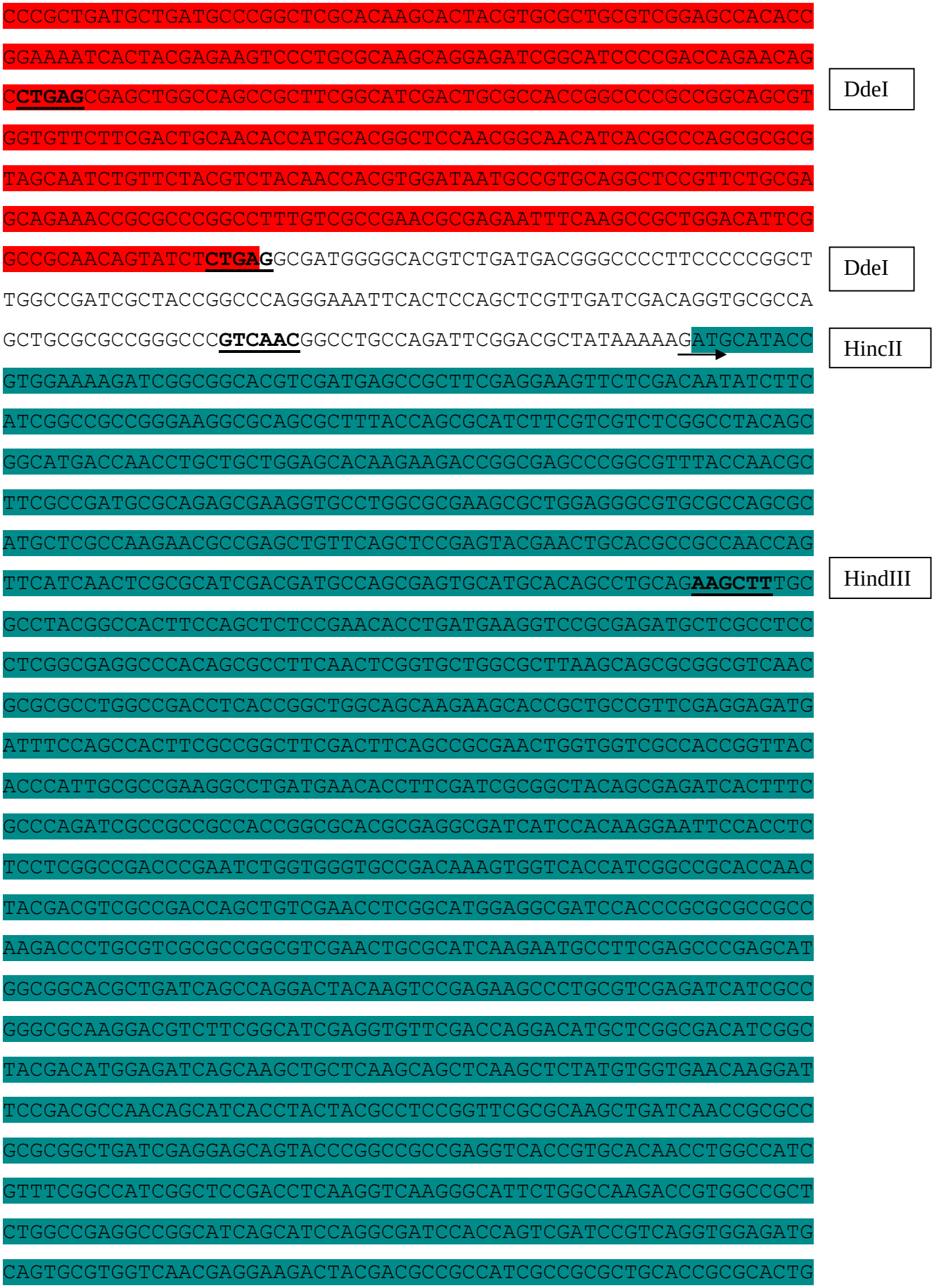
DdeI
DdeI
HincII
HindIII

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AGACGCGCGAGGCGCCCCATCGCTCCCCCATGAAGCAGGAGTTGCCATGTTCCCCGAGTA

CCGCGATCTGATCACCCGTCTCAAGGCTGAAAACAAGACCTTTGCCCGCCTGTTCGACGA

GCACAACGACCTCGACCAGCGGGTGAAGAACATCGAGGCGCACATCGTGCCGGGCACCGA

CAGCGAGGTGGAAAACCTGAAGAAGCAGAAGCTGCAGCTCAAGGATCGCCTCTACGACAT

GCTCAAGGAGGCATCCGTCGCCTGACCCTCTGCGCGCCCCGCTCGCGGGGCGCTGCCTGC

CGCGTCATTGCGGACAACGCTCGACGCGCAATCGCCGGGCAAGCGCAGTAAAATGCCGCG

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GAAGGTCGGCATCGACGAGGCCCACGCGCTGGGCAAGCAGTTCTACGTGGTGGTCAACAT

CGCCCCGCACAACGCCAAGCTCAAGACCTTCATCAAGGACCTGGAGCCGGTGGTAGCGAT

GGG**TCCGGA**CGCGCTGATCATGTCCGACCCGGGCCTGATCATGCTGGTGCGCCAGCATTT

TCCGCAGCAGACCATCCACCTCTCGGTACAGGCCAATGCGGTGAACTGGGCGACGGTGAA

GTTCTGGGAAAGCCAGGGCGTCTCCCGGGTGATCCTTTCCCGCGAGCTGTCGCTGGAGGA

GATCGGCGAGATACGCGAGCAGGTGCCGGGCATGGAGCTGGAAGTGTTCGTCCATGGTGC

BspE

Appendix 4 – The DNA sequence of the expected pCrc_32_OectA_OectC plasmid

Red: *rbcL* region = (The hole sequence 161 bp, 5' UTR and promoter for *OectA* 137 bp)

Beige: *atpH* region = orange (5' UTR + promoter for OectC 93 bp)

Green: *OectC* = green (401 bp)

Turquoise: *petA* region = light blue (3'prime end for OectC 72 bp)

Yellow: *OectA* = yellow (582 bp)

Blue: *psaB* region = dark blue (3' prime end for OectA 348 bp)

Brown: BamHI-EcoRI = brown (3883 bp, not counting the atpB gene)

Rosa: *atpB* = purple (1476 bp)

Light grey: pUC8 = light grey (2849 bp)

Dark grey: Extra sequences = dark grey

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TAAATACCATGTGTGTATTCTAAATAGGTTTATTGGGGTACTTTATTTTACACTTTATTTTA
CACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACA
CTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACT
TTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTTATTTTACACTTT
TTTAATATGAATTAAATTAAATTAATTTAATTTAATTTAAGAGAACCAATTACTGGTATAAT
TTGGAAAATAAAATACATTTAATCTGGTAATTTATTTGGTAATTTGGTCAAAATTATAATAG
TTTTTATTTGATTTTTTTGAATTGAATTTAAAAAGACATCCCCTTACAGTATTCTTGCAAAC
CTTACGGGACAATAAATAAATTTGTTGCCTGCCAACTGCCGATATTTATATACTAGGACGTC
CCCTTCCCCTTACGGGATATTTATATACTCCGAAGAGCAAGTAAACTTAGGGATTTTAATGC
AATAAATAAATTTGTCCCCTTACGGGAATATAAATATTAGTGGACGTCCCCTTCGGGCAAAT
AAATTTTAGTGGCAGTTGCCTCGCCTATCGGCTAACAAGTTCCTTCGGAGTATATAAATATA
GGACGTCAGTGGCAGTTGCCTGCCAACTGCCTATATTTATATACTGCGATAAACTTTAGTTG
CCGAAGGGGTTTACATACTCCGAAGGAGGAAGCAGGCAGTGGCGGTACCACTGCCACTAAAA
TTTATTTGCCTCCTAACGGAGCATTAAAATCCCGAAGGGGACGTCCTGCCAACTGCCCTTGC
CGCAGTATTAACATCCTATATTTATATACTCCGAAGTATATTTATATGCTTCCCCTTCCTTC
GGGTATATAAATATTGGGCAAGTAAACTTAGGAGTATATAAATATAGGACGCCAGTGGCAGT
GCCCCCTCGAG

Appendix 5 – 1kb Plus DNA ladder

1kb Plus DNA ladder

0.7 µg/lane

0.9 % agarose gel

Stained with ethidium bromide

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