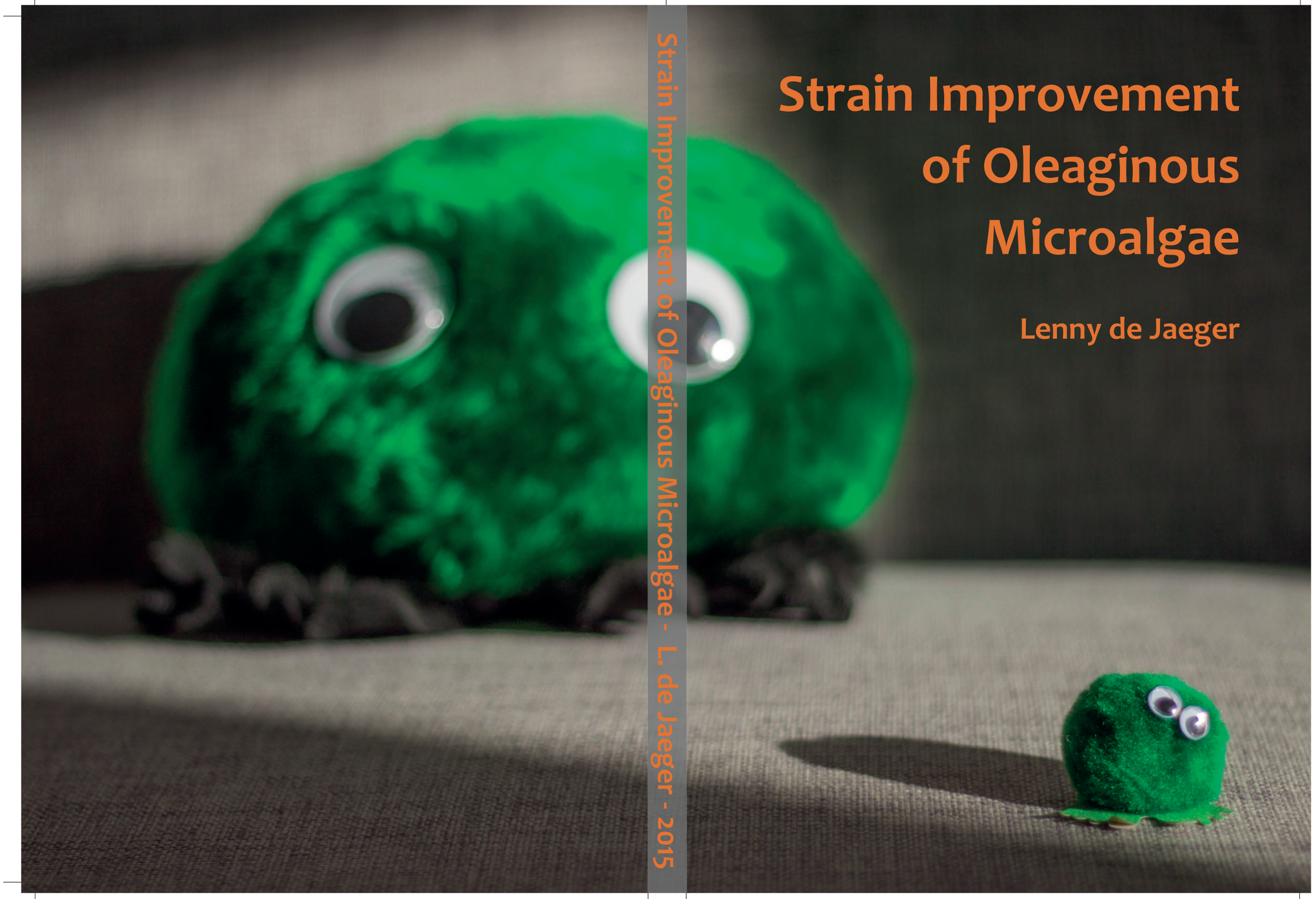

Strain Improvement of Oleaginous Microalgae

Lenny de Jaeger

Strain Improvement of Oleaginous Microalgae - L. de Jaeger - 2015

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Strain Improvement of Oleaginous Microalgae

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Thesis

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"It is hard to make that boat go as fast as you want to. The enemy, of course, is resistance of the water, but that very water is what supports you and that very enemy is your friend."

- George Yeoman Pocock -

Contents

Chapter 1

Introduction and thesis outline

1.1 Introduction

The increasing world population and living standards have enlarged the demand for food, feed, and for chemicals for industrial purposes. Traditional fossil fuel based commodities need to be replaced, not only because these resources are finite, but also to relieve the impact of carbon emission and pollution, resulting from fossil fuel derived processes. Much attention is on using plants to produce sustainable, renewable alternatives to petrochemical based processes. Palm oil is the crop with the highest lipid yield known today (Edwards et al., 2014). The production of palm oil has increased tremendously in countries like Malaysia and Indonesia over the past 40 years. In Malaysia alone, the land used for palm oil production increased from 54,000 hectares in 1960 to 4.05 million hectares in 2005, resulting in a production of 94,000 tons palm oil in 1960 rising to 15 million tons in 2005 and almost 20 million tons in 2014 (Basiron, 2007; MPOB, 2015). This leap in palm oil production has caused deforestation on a very large scale, which is still ongoing (Wicke et al., 2011). Besides plant derived oils for consumption and biodiesel production, plant crops need to provide our carbohydrate and protein supply as well. In the past decades, researchers have been looking for an alternative agricultural crop that has a high productivity and does not have such a large impact on available cropland. Microalgae are a promising candidate to fulfill this role in the future, because of their high biomass and lipid productivity (Chisti, 2007). In addition to lipids, microalgae can produce proteins and carbohydrates for the use in food and feed. Microalgae use the energy of the sun to convert carbon dioxide into sugars, which are the building blocks for many molecules,

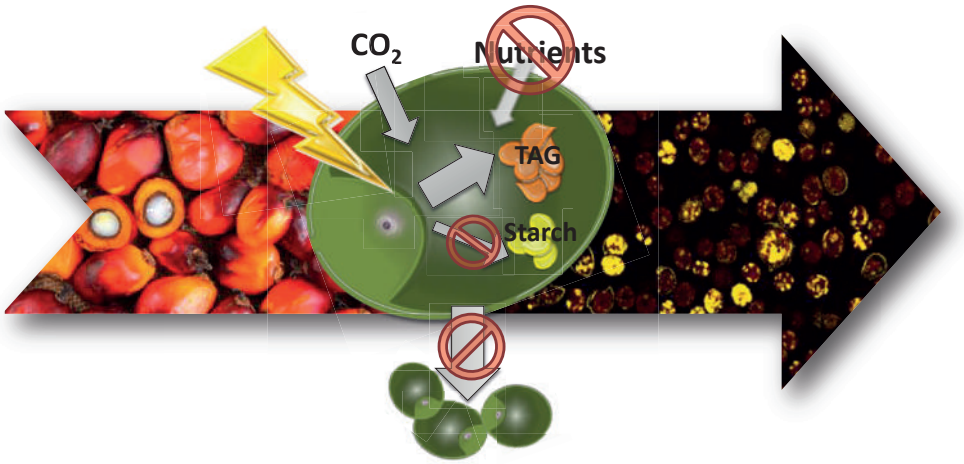

Figure 1.1 From Palm oil to microalgae derived oil. Palm oil kernels (left) and *Scenedesmus obliquus* (right) with TAG lipid bodies (yellow fluorescence). Light energy is used to produce biomass under nutrient replete conditions. When nutrients (nitrogen) are depleted from the medium, cell growth is restricted, and light energy is used to make molecules such as TAG and starch. When starch accumulation is blocked, the carbon allocation towards TAG is increased.

such as lipids and starch (Figure 1.1). To replace current vegetable oil sources, microalgae need to accumulate large amounts of the neutral lipid triacylglycerol (TAG), which could be applied for edible oils as well as for biodiesel production. A great advantage of microalgae over traditional production crops is their independence of fertile land, because they can be cultivated in photobioreactors and open ponds that can be placed on non-arable land. Currently, microalgae derived products have proven to be functional and a potential replacement for conventional crops. However, microalgae derived products, especially lipids, are not economically feasible yet, with the exception of high value products, such as carotenoids and polyunsaturated fatty acids (PUFAs). In order to make microalgal derived products a reality we need to decrease the production costs by smart technological solutions, biological understanding and metabolic engineering.

Over the years there has been an enormous improvement in the field of genetic modification in bacteria, yeast, fungi, mammalian cells and (higher) terrestrial plants. However, less improvement has been made on the genetic engineering of microalgae, despite the fact that there has been a strong interest in microalgae for the production of different products. But, in addition to the improvement of the culture technology, strain improvement is also required. The aim of the work described in this thesis was to explore the lipid production mechanism in microalgae and, using this knowledge, to improve microalgal lipid productivity and lipid composition using strain improvement strategies. In this thesis the term 'microalgae' refers to eukaryotic microalgae, as only eukaryotic algae produce triacylglycerol (Wijffels et al., 2013), which is the lipid source that can be used for edible oil and biofuel production.

1.2 Strain improvement

In most industrial biotechnological processes wild type strains have been improved by breeding, mutation and selection, or metabolic engineering. So far, algal production processes have been based on wild type strains and improvement of microalgal strains is still in an early stage of development. In all expression systems there is a model organism that allows detailed study of the behavior of the host and to gain understanding on the metabolism and biology of the organism. *Escherichia coli* and *Arabidopsis thaliana* are the model strains for bacteria and higher plants respectively, and *Chlamydomonas reinhardtii* is the model strain for green microalgae. *C. reinhardtii* was the first green microalga to be sequenced (Merchant et al., 2007) and decades of research on photosynthesis in this species have improved our understanding of green microalgae.

There are several ways to improve traits in microalgae. A well-established method is using **mutagenesis**, a method in which mutations are randomly created in the genomic DNA of the microalgae which may result in changes in the phenotype. There are several

different mutagens that can be used to induce mutations in the genome, such as UV light and several chemicals. The mutation can reduce or completely disrupt the functionality of an enzyme, which in turn can influence metabolism. After exposure to a mutagen, surviving cells will need to be screened to select the mutants that have the required phenotype. This is an elaborate process that requires the right selection or screening characteristic.

A more direct approach would be using direct **genetic engineering** techniques, which is based on the introduction and integration of exogenous DNA into the host cell. There are several methods that can be used to deliver the DNA with the gene of interest in the target species. The most commonly used methods are described below and summarized in table 1.1.

Table 1.1 Different methods to deliver exogenous DNA

Method	Mechanism	Comment
Particle bombardment	DNA coated particles fired into cells	Complex equipment
Silicon carbide whiskers	Fibers penetrate cell membrane when vortexed	Low efficiency
Glass beads	Permeabilize cells using glass beads and vortexed	Protoplasts only
Electroporation	Electric pulse introduces transient pores	Complex equipment
Liposomes	DNA coated with lipids fuse with cell membrane lipids	Protoplasts only
Agrobacterium tumefaciens	Bacterium uses DNA delivery mechanism to infect target cells	Laborious procedure
Virus	Cells are infected by a virus with exogenous DNA	Limited experience
Microinjection	Direct DNA delivery inside the target cell	Complex equipment

1.3 Introduction of exogenous DNA into the host cell

To get exogenous DNA into the cell of a microalgal species is very dependent on the exterior design of the cell wall. Some species have robust and tough cell walls, which make DNA delivery difficult, while others completely lack a cell wall. The large diversity in microalgal cell wall composition is caused by the wide spread distribution of microalgae over very different ecosystems. All these different ecosystems require different biological traits to survive under these conditions. This resulted in different specialized cell wall designs for different microalgal species. Therefore, diverse strategies are needed to transform different microalgal species. The following methods are applied in microalgal genetic engineering.

A widely used mechanical DNA delivery system is **biolistic transformation** or micro-particle bombardment. Gold or tungsten particles are coated with DNA and shot into microalgal cells under vacuum using a helium pressurized gun. The method can be used on a wide range of microalgal species, including robust silicon covered diatoms, since the physical barriers such as the cell wall can be overcome (Apt et al., 1996). In addition to nuclear transformation, chloroplast or mitochondrial genomes can be targeted, which is not possible with any other method. A major drawback of this method is the laborious protocol and the need for specialized and expensive equipment.

A method widely adopted in transforming microalgal cells is **agitation with glass beads**. Microalgal cells are vortexed in the presence of exogenous DNA and glass beads. The glass beads create pores in the cell membrane through which the DNA can enter the cell. The addition of the membrane fusion agent polyethylene glycol (PEG) can enhance the efficiency of transformation (Kindle, 1990). This method has been applied to many microalgal species and was first described for *C. reinhardtii* (Kindle, 1990). However, the method is more efficient for microalgal cells that lack a cell wall. Some species, such as *Dunaliella salina*, lack a cell wall by nature, but for other species the cell wall must be removed using an enzyme cocktail to generate protoplasts. In the case of *C. reinhardtii*, cell wall less mutants were created, which can be transformed very efficiently using the glass bead method, but as a result are shear sensitive and therefore not suitable for large scale cultivation.

A method based on the mechanical disruption of the cell wall and cell membrane of microalgae is using **silicon carbide (SiC) whiskers**. Microalgal cells are supplemented with elongated SiC fibers and DNA then the mixture is mixed on a vortex. DNA can penetrate the cell wall through the small holes that are created in collisions between the microalgal cells and the 10-80 μm long SiC whiskers. The main advantages of the SiC mediated transformation are the easy, quick and inexpensive procedure and there is no need for complex equipment. The main disadvantages of the method are the low transformation efficiency, due to very high cell mortality, and the need for strict protection during lab work, since inhalation of the fibers can lead to serious illnesses. The efficiency of SiC mediated transformation depends on the size of the SiC whiskers, the vortex time, the shape of the mixing tube and the cell wall characteristics of the microalgae. Although, many transgenic plants have been created using SiC mediated transformation (Kaeppler et al., 1990; Nagatani et al., 1997; Petolino et al., 2000), and the method proved to work in microalgae (Dunahay, 1993), it is not applied often to transform microalgae.

The most widely used method to introduce foreign DNA into microalgae is the physical DNA delivery method **electroporation**. Transient pores are introduced in the cell membrane, by reshuffling the membrane lipid molecules, by placing an electric charge over

the cell. This opens up a transient pore which fills with water and functions as a channel through the cell membrane, enabling cellular introduction of large highly charged molecules, such as DNA, which cannot passively diffuse across the hydrophobic bilayer of the cell. Electroporation is widely used because it is a relatively simple, time efficient and inexpensive method.

Adherence of the exogenous DNA to the cell membrane of the host organism can increase the efficiency of DNA uptake into the cell upon pore formation. Most microalgae have an external cell wall and therefore the adherence of the exogenous DNA to the cell membrane is not very efficient. This can be overcome by generating protoplast cells by using enzymes that degrade the cellulose rich cell wall, or by using cell wall less mutants (Shimogawara et al., 1998). Call wall less mutants, can be transformed using electroporation more easily, since the pore formation is more efficient compared to cell wall containing strains. The electroporation settings need to be optimized for every individual species to obtain successful transgenic microalgae. A higher voltage will increase the degree of permeability, but will reduce viability of the cells as well (Azencott et al., 2007). Electroporation has already been applied successfully to a number of microalgal species including *C. reinhardtii*, *Nannochloropsis sp.*, *Chlorella vulgaris* and many more (Chow and Tung, 1999; Coll, 2006; Kilian et al., 2011; Shimogawara et al., 1998).

***Agrobacterium tumefaciens* mediated transformation** makes use of the biological infectious mechanism of the gram-negative bacterium *A. tumefaciens*. In nature this bacterium induces tumor formation in plants by inserting a small stretch of transfer DNA (T-DNA) in the genome of the plant. This piece of DNA is maintained on a plasmid (Ti-plasmid) and contains a set of genes to enable successful integration into its host genome. This mechanism has been adopted and domesticated in such a way that the genes of interest can be inserted in the host organism using *A. tumefaciens* as delivery vehicle, but the tumor inducing genes are omitted from the plasmid. The gene of interest is flanked by a left and right border and the DNA between the left and right border is cut out of the plasmid, inserted into the cell and then transported to the cell nucleus where it is integrated in the genomic DNA. *A. tumefaciens* mediated transformation was first introduced for plant cells but has been shown to work in other organisms such as algae as well (Kumar et al., 2004).

It has been shown that *A. tumefaciens* mediated transformation yields higher amounts of transformed cells compared to using other techniques and, in addition, the transformants are also more stable (Qin et al., 2012). The method requires no special equipment, but does require a laborious microbiology based procedure, since the level of infectivity of *A. tumefaciens* depends on many factors such as pH, temperature and the presence of plant hormones such as acetosyringone.

Methods that are used less frequently or are in development stage are viral-mediated transformation, microinjection and lipofection. Recombinant eukaryotic algal viruses, although not applied widely, is a method to transform microalgae for which the potential has been shown in brown algae and certain *Chlorella* species (Henry and Meints, 1994; Van Etten and Meints, 1999). Microinjection is a very efficient mechanical approach to penetrate microalgal cells with exogenous DNA, where DNA is injected directly into the target cells. A drawback of this method is that it requires specialized equipment and the microalgal cells need to be immobilized to enable exact placement of the needle. *Acetabularia mediterranea* was transformed using microinjection, but there are few other examples (Neuhaus et al., 1986). A biochemical method that is often used in mammalian cell and plant transformation, is the use of liposomes. Liposomes are microscopic spherical vesicles built up from phospholipids, which can be loaded with molecules, such as DNA. The transfection, or lipofection, occurs when the liposome fuses with the cell membrane releasing the exogenous DNA in the host cell. This method requires cell wall less mutants or microalgal protoplasts and therefore cannot be applied to all species directly. There are no examples of successful transgenic microalgae which are obtained using lipofection.

1.4 Incorporation of exogenous DNA in the genomic host DNA

Inserting the DNA inside the microalgal cell is just the first step in obtaining transgenic microalgae. When the exogenous DNA has entered the cell, it can be randomly incorporated in the genomic host DNA. The stretch of exogenous DNA is typically composed of a construct containing the gene of interest and a selection gene. The selection gene enables the selection of cells that have successfully incorporated and expressed the exogenous construct. A method that is often used is the reintroduction of a gene which is nonfunctional in the target strain. For example, nitrate reductase deficient mutants cannot grow on nitrate as sole nitrogen source and need ammonium instead. The mutants can be transformed with a native nitrate reductase, which enables selection on nitrate (Dawson et al., 1997; Kindle et al., 1989). Alternatively, antibiotic resistance genes are used, which make the target cells insensitive to the toxic antibiotic upon successful integration and expression of the construct.

Since the exogenous DNA is inserted randomly in the target genomic DNA, each transformant might have a different level of expression, depending on the position of the integration in the genome. Several other factors have been determined that can influence the expression of heterologous genes in microalgae (figure 1.2). An important factor in the ability of a transgenic microalgae to express the transgene, is the similarity between the codon bias of the transgene and the host microalgae. This bias is caused by the fact that microalgae have a relatively high GC content compared to other organisms. The

nuclear genome of *C. reinhardtii*, for instance, has a GC content of over 65% (Jarvis et al., 1992; Nakamura et al., 2000). Therefore, codon optimization is required when transgenes are used from species with a different codon bias.

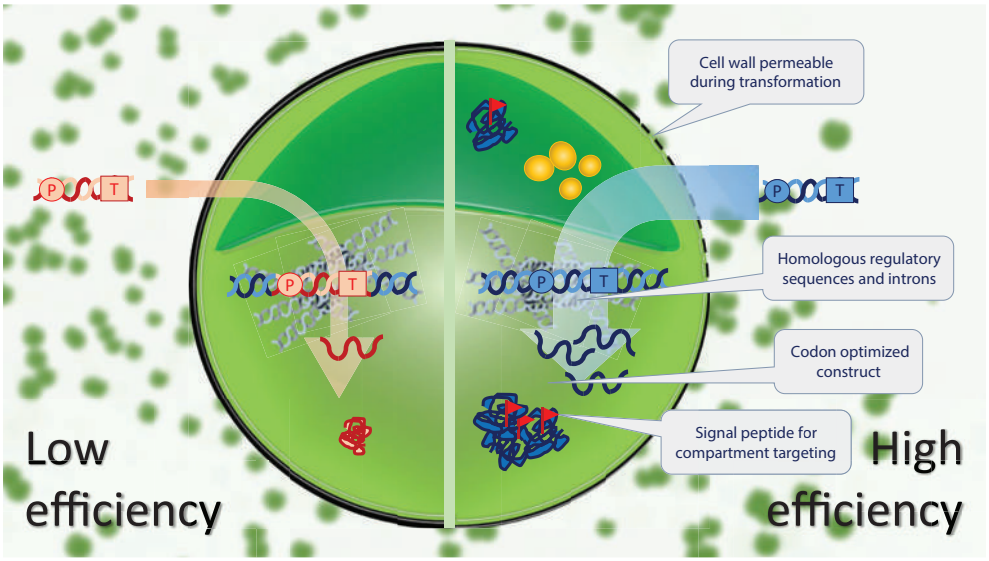

Figure 1.2 Bottlenecks that are encountered in expressing transgenes in microalgae. Example is given for a product of interest produced in the chloroplast.

The transcription of genes is initiated by a region of DNA upstream of the transcription start site of a gene, the promoter. The promoter facilitates the attachment of RNA polymerase. Promoters can be used to direct the level of transcription of the gene by regulatory regions such as enhancers and silencers. Transgenes are accompanied by promoter sequences, which are often of heterologous, mostly plant, origin. The use of native homologous promoters is desired, because the promoter will be recognized more effectively by the native RNA polymerase, and often a promoter is chosen which is associated with genes that are highly expressed. For efficient expression of transgenes, endogenous promoters are used, such as the promoter from ribulose bisphosphate carboxylase (RBCS2), or the fusion promoter of heat shock protein 70A and RBCS2, which enables induction of the expression of the transgene on demand by applying a heat shock (Schroda et al., 2000). The transgene is flanked by a promoter upstream and a terminator downstream of the gene. The terminator sequences is important for the stability of the mRNA (Cerutti et al., 1997; Lumbreras et al., 1998). Finally, the stability and efficiency of transgene expression can be enhanced by introducing introns in the gene, especially in case when the transgene is from prokaryotic origin, which do not contain introns (Lumbreras et al., 1998).

The efficiency of transgene expression in microalgae is not yet optimal. This is due to the limited knowledge on gene regulation in microalgae, and the limited number of sequenced genomes of industrially relevant microalgal species. Therefore, efficient promoter sequences and essential expression enhancing introns have not been identified in many species. This is rapidly changing and, in line with this, the genetic engineering revolution is developing in the microalgal community.

1.5 Thesis outline

The main goal of the work described in this thesis was to increase the lipid productivity of microalgae and to improve the fatty acid profile of microalgal lipids for industrial applications. This is needed to make microalgal lipid production economically feasible and competitive with conventional lipid producing crops. To improve the lipid productivity of microalgae, strain improvement strategies such as metabolic engineering and mutagenesis are needed.

To get more insight in the lipid accumulation mechanism of microalgae and to define targets for future strain improvement strategies, transcriptome sequencing of the oleaginous microalgae *Neochloris oleoabundans* was used. This oleaginous species was selected, since it can tolerate both fresh and salt water. This is an interesting feature to reduce the fresh water footprint in large scale production of microalgae. In **chapter 2** *N. oleoabundans* was cultivated in photobioreactors to ensure defined conditions and reproducible cultivation. Cultures grown in saline or fresh water, under either nitrogen replete or depleted conditions, were compared on major biomass composition and the mRNA was sequenced. In addition to the lipid production pathway, the salt resistance mechanism was studied in detail, which could be applied to improve salt resistance in other industrially relevant microalgal strains.

An alternative, non-GMO, approach to obtain improved microalgal strains is by using mutagenesis. In **chapter 3**, UV mutagenesis was used to create starchless mutants. The oleaginous microalgae *Scenedesmus obliquus* was selected as target strain because of its high lipid productivity and capability of maintaining its photosynthetic efficiency upon nitrogen starvation. Due to its rigid and robust cell wall, no transformation approach was available for this species and therefore mutagenesis was used. Starchless mutants of *S. obliquus* were isolated by screening for reduced starch content using Iodine vapor staining. The isolated starchless mutants all showed an increased triacylglycerol productivity, since the photosynthetic derived sugars could not be incorporated into starch and therefore were used to produce extra triacylglycerol molecules under nitrogen depleted conditions. Starchless mutant 1 (Slm1) showed the highest lipid productivity and triacylglycerol content.

In **chapter 4** the Slm1 strain was compared to the wild type strain. The use of photobioreactors enabled frequent biomass sampling over time to study the differences in biomass composition between the two strains under defined light conditions. The results were used to get insight into the carbon partitioning, and the effect of the starch knockdown on the physiology of the Slm1 strain compared to the wild type. In **chapter 5** the transcriptome under nitrogen replete conditions of both the wild type and the Slm1 mutant was sequenced. Under these conditions, starch is produced in the wild type and the starch pathway was studied at the transcriptional level. UV mutagenesis introduces transversions in the genome resulting in single nucleotide polymorphisms. A single nucleotide polymorphism was identified that caused the knockdown of the starch pathway and this was confirmed by PCR sequencing. **Chapters 4 and 5** provide understanding of carbon partitioning in oleaginous microalgae, leading to promising targets for future genetic engineering approaches to increase triacylglycerol accumulation in microalgae.

For successful transformation of microalgae, we need insight in the parameters that result in permeabilization of the cells to enable the uptake of exogenous DNA. When cells are permeabilized too much, the cells will rupture and will not survive the procedure. Thus, there is a delicate balance between successful transformation and cell death, which is different for each species depending on the cell wall composition. In **chapter 6** a method is presented to quantify the permeable and viable cell population within a transformed cell population, using fluorescent dyes and high throughput analysis on a flow cytometer. This method can be used to successfully develop various transformation strategies for industrially relevant microalgae that are not studied in much detail.

The value of microalgae derived lipids can be increased when these lipids are customized to certain specific industrial requirements. Since the toolbox for transforming oleaginous microalgae is not yet well established, we used a lipid accumulating starchless mutant of the model species *C. reinhardtii* BAFJ5 as our host in **chapter 7**. Artificial microRNA was used to silence the stearoyl-ACP desaturase gene in the starchless mutant of *C. reinhardtii*. This resulted in an increase in the stearic acid content in the triacylglycerol molecules, which is of economic relevance, because of the improved structural properties.

In **chapter 8**, an outlook is given on microalgal strain improvement strategies for the future, reflecting on the results obtained in this thesis. Also a roadmap is suggested to get genetically modified microalgal derived products on the market.

Chapter 2

Neochloris oleoabundans is worth its salt: transcriptomic analysis under salt and nitrogen stress

Submitted for publication as:
Neochloris oleoabundans is worth its salt:
transcriptomic analysis under salt and nitrogen stress
Lenny de Jaeger, Benoit M. Carreres, Jan Springer, Dirk E. Martens, Peter J. Schaap,
Gerrit Eggink and René H. Wijffels

Neochloris oleoabundans is an oleaginous microalgal species that can be cultivated in fresh water as well as salt water. This gives opportunities for reducing production costs and fresh water footprint for large scale cultivation. This is the first report that provides insight in the saline resistance mechanism of a fresh water oleaginous microalgae. To better understand the osmoregulatory mechanism of *N. oleoabundans*, the transcriptome was sequenced under four different conditions: fresh water nitrogen replete and depleted conditions, and salt water (525 mM dissolved salts) nitrogen replete and depleted conditions. In this study several pathways are identified that are responsible for salt water adaptation of *N. oleoabundans* under both nitrogen replete and depleted conditions. Proline and the ascorbate-glutathione cycle seem to be of importance for successful osmoregulation in *N. oleoabundans*. NMR spectroscopy revealed an increase in proline content in saline adapted cells. This was supported by up regulation of the genes involved in proline biosynthesis. Additionally the lipid accumulation pathway was studied to gain insight in the gene regulation in the first 24 hours after nitrogen was depleted. Oil accumulation is increased under nitrogen depleted conditions in a comparable way in both fresh and salt water. The mechanism behind the biosynthesis of compatible osmolytes in *N. oleoabundans* can be used to improve *N. oleoabundans* and other industrially relevant microalgal strains to create a more robust and sustainable production platform for microalgae derived products in the future.

2.1 Introduction

Sustainable and renewable production of energy and food for an increasing world population is an enduring challenge in present-day research. This challenge must be addressed with urgency, because of the world's dependence on limited fossil fuels and the increase in living standards of emerging economies. Renewable energy platforms based on oleaginous agricultural crops such as rapeseed, palm oil, corn, and soybean are being studied. Although these crops are considered renewable and bio-based, they increase the competition for food, fresh water, the amount of available arable land and results in deforestation to create plantations. Ideally we would use land that is not suitable for traditional agriculture such as salt contaminated land or very dry areas like deserts for this purpose. A promising alternative feedstock compared to traditional crops for the production of oil are microalgae (Chisti, 2007; Wijffels and Barbosa, 2010). Microalgae can produce high amounts of neutral lipids, triacylglycerol (TAG), when exposed to unfavorable growth conditions. TAG can be easily converted in biodiesels by methylation, which results in a pure and clean fuel that can replace petroleum-derived fuels (Chisti, 2007). The TAG molecules can also be used directly in the food and feed industry as a sustainable vegetable oil replacement. For an acceptable sustainable production process with a reduced fresh water footprint the use of marine or salt tolerant microalgal species is essential.

Most organisms are not able to cope with a shift in osmotic pressure when the environment is changed from fresh water to salt water and their growth will be compromised. Some organisms are able to adapt to such changes. Plants have developed different strategies to deal with osmotic stress. In addition to the strategies that involve structural traits such as waxes and adaptation of flowering time to the right conditions and moment, plants can also regulate their osmotic homeostasis by actively excluding salts from the cell to maintain water absorption. Another strategy involves the accumulation of certain compatible organic osmolytes. A few examples of these compatible osmolytes are proline and glycine betaine (Ashraf and Foolad, 2007), cyclic polyols such as D-pinitol (Streeter et al., 2001), and sugars such as sucrose hexoses and sugar alcohols (Hoekstra et al., 2001).

Prokaryotic microalgae are known to accumulate sucrose or α-glocosylglycerol under salt stress conditions (Desplats et al., 2005). In eukaryotic microalgae there are some strategies that can be found to overcome salt stress. Some species of microalgae are known to be able to survive low levels of saline environments. Known mechanisms for salt tolerance are glycerol production (Hellebust and Lin, 1989), sucrose production (Band et al., 1992), and amino acid accumulation (Mastrobuoni et al., 2012).

The oleaginous salt tolerant microalgae *Neochloris oleoabundans* is a very interesting candidate that could be used for lipid production (Band et al., 1992; Gouveia et al., 2009; Santos et al., 2012). *N. oleoabundans* can accumulate TAG at up to 44% of its dry weight resulting in a maximal productivity of 164 mg L^{-1} day^{-1} (Breuer et al., 2012; Griffiths and Harrison, 2009). The harsh desert conditions from which this oleaginous microalgae was isolated (Chantanachat and Bold, 1962), forced *N. oleoabundans* to be a highly flexible species to deal with the daily salt, drought and temperature stresses during the hot days and cold nights. These properties, combined with the high growth rate of *N. oleoabundans* (μ 2.2 D^{-1}) (Gouveia et al., 2009), and its resistance to highly alkaline conditions (up to pH 10) (Santos et al., 2012), which enhances the CO_2 transfer and reduces risk of contamination, makes it a very interesting candidate for sustainable oil production.

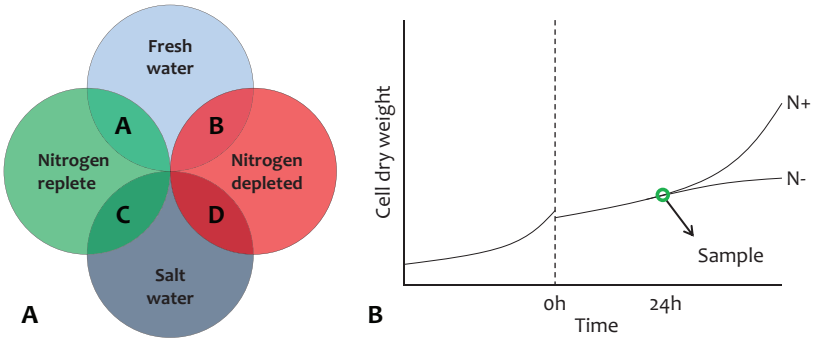

Figure 2.1 Set up of the cultivation experiment (A) Representation of the four different environmental conditions that were examined in this study (B) Cultivation set up. Two cultures were grown in fresh water in nitrogen replete conditions. The dotted line indicates the moment of medium replacement. One culture remained nitrogen replete, the other culture was exposed to nitrogen depleted medium. The same regime was applied to salt water adapted cultures. The whole experiment was conducted in duplicate. This method results in comparable growth, lighting and nitrogen depletion conditions.

In this study, the mechanism the fresh water green microalga *N. oleoabundans* uses to cope with saline growth conditions is assessed using transcriptome sequencing. The transcriptome of this versatile organism was studied under four different conditions (Figure 2.1), to get insight in the pathways that are involved in salt resistance and lipid accumulation. To our knowledge, *N. oleoabundans* is the first fresh water microalgae, studied on transcriptomic and metabolite level, which is able to alleviate the osmotic stress under salt water conditions. We will discuss and compare the different pathways that are involved in the saline and nitrogen stress response. These findings can be used to get a better understanding of these processes and to define targets for new strategies to enhance microalgal strains to increase lipid productivity in the future.

2.2 Material and Methods

2.2.1 Strain, medium and pre culture

Neochloris oleoabundans UTEX 1185 (University of Texas, Austin, USA) pre-cultures were maintained in 100 mL filter sterilized fresh or salt water medium in 250 mL Erlenmeyer shake flasks. The fresh water medium consisted of: KNO_3 50.5 mM; Na_2SO_4 4.6 mM; HEPES 100 mM; $MgSO_4.7H_2O$ 1 mM; $CaCl_2.2H_2O$ 0.5 mM; K_2HPO_4 4.1 mM; $NaHCO_3$ 10 mM; NaFeEDTA 0.14 mM; $Na_2.EDTA.2H_2O$ 0.4 mM; $MnCl_2.4H_2O$ 96 µM; $ZnSO_4.7H_2O$ 21 µM; $CoCl_2.6H_2O$ 6 µM; $CuSO_4.5H_2O$ 6.6 µM; $Na_2MoO_4.2H_2O$ 0.5 µM; biotin 0.2 µM; vitamin B1 7.4 µM; vitamin B12 0.2 µM. The same medium was used for salt water with the following modifications: NaCl 448 mM; $MgSO_4.7H_2O$ 5 mM; $CaCl_2.2H_2O$ 2.4 mM. The pH for both media was set to pH 7.5 using NaOH and the medium was filter sterilized (0.2 µm) prior to use. When nitrogen depleted conditions were applied the KNO_3 was omitted and replaced by an equimolar amount of KCl. In the shake flasks, pH was buffered using HEPES, while in the bioreactor HEPES was omitted and the pH was controlled by CO_2 addition.

2.2.2 Reactor design

All experiments were performed in flat-panel, Algaemist airlift-loop photobioreactors (Breuer et al., 2013b). The reactors, with a working volume of 0.38 L, were kept at 25°C and pH controlled using on demand CO_2 (pH 7.5). Light was supplied continuously and the incident light intensity was adjusted to maintain constant average light intensity (80 µmol m^{-2} s^{-1}). The cultures were inoculated at a biomass concentration of 0.15 g L^{-1} in either fresh or salt water medium. When the biomass concentration reached approximately 4.5 g L^{-1}, the cultures were harvested and washed with either salt or fresh water medium containing nitrogen or no nitrogen. The cells were cultivated for 24 hours before sampling. Samples for dry weight, total fatty acid triacylglycerol, starch, NMR analysis, and RNA extraction were taken. All reactors were run in duplicate, resulting in 8 individual samples for four different conditions.

2.2.3 Determination of dry weight concentration

Dry weight concentrations were determined on biological replicates. Around 1.5 mg of biomass was filtered through pre-dried (100°C overnight) and pre-weight Whatman glass fiber filter paper (GF/F; Whatman International Ltd, Maidstone, UK). The filter was washed with 50 mL of filtered demineralized water supplemented with an equimolar concentration of NH_4HCO_2 to prevent osmotic shock and subsequently dried overnight at 100°C before weighing.

2.2.4 Starch analysis

The starch content was analyzed using the Total Starch assay (Megazyme International, Wicklow, Ireland) following the protocol described previously (de Jaeger et al., 2014).

The method is based on enzymatic degradation of starch to glucose monomers by α-amylase and amyloglucosidase enzymes and measuring glucose monomers in a spectrophotometric-based assay for quantification against a D-glucose calibration control series at a wavelength of 510 nm.

2.2.5 Total fatty acid analysis

Total fatty acid (TFA) extraction and quantification were executed as described by Breuer *et al*. (Breuer et al., 2013a) with the following adjustments. Around 5 mg of pellet was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) and lyophilized overnight. Freeze-dried cells were disrupted by a 30 min bead beating step in the presence of a chloroform:methanol mixture (1:1.25) to extract the lipids from the biomass. Tripentadecanoin (T4257; Sigma-Aldrich, St Louis, MO, USA) internal standard was added to the extraction mixture to enable fatty acid quantification. For TFA analysis, samples were directly methylated (see below). For TAG analysis directly after the TFA extraction, the chloroform methanol mixture was evaporated under N_2 gas and the TFA fraction was dissolved in 1 mL hexane and separated based on polarity using a Sep-Pak Vac silica cartridge (6 cc, 1,000 mg; Waters, Milford, MA, USA) prewashed with 6 mL of hexane. The neutral TAG fraction was eluted with 10 mL of hexane-diethyl ether (87:13% v/v). The polar lipid fraction containing the glycolipids and phospholipids remained in the silica cartridge. Methylation and quantification of the fatty acids to fatty acid methyl esters (FAMEs) was performed as described by Breuer (Breuer et al., 2013a).

2.2.6 Bodipy staining

The presence of neutral lipid bodies in *N. oleoabundans* was measured by staining the cells with the fluorescent dye BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazasindacene; Invitrogen Molecular Probes, Carlsbad, CA). An aliquot of 200 µL of cells was incubated for 10 minutes with 4µL of a 40 µM BODIPY stock solution (in 0.2% (v/v) DMSO) and subsequently studied using a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany), using a 488 nm Argon Laser (Klok et al., 2013a).

2.2.7 NMR analysis

NMR analysis was performed according to the protocol described by Kim et al. (Kim et al., 2010a) with the following modifications. Freeze dried microalgal biomass (20 mg) was dissolved in 0.5 ml of 50% deuterated methanol in buffer (90 mM KH_2PO_4 in D_2O) containing 0.05% trimethyl silyl propionic acid sodium salt (TMSP, w/v) as internal standard. To effectively extract the metabolites, the cell suspension was vortexed and ultrasonicated for 10 min and centrifuged at 17,000xg for 5 min. From the supernatant, 300 µl was used to perform NMR analysis. The NMR analysis and data analysis was carried out as described by Kim et al. (Kim et al., 2010b). The NMR plots can be found in Appendix figure 2.A1.

2.2.8 RNA extraction

Samples for RNA isolation were immediately processed after sampling and kept on ice. Cells were collected by centrifugation at 4.000xg at 0°C for three minutes and immediately frozen in liquid nitrogen before storage at -80°C until further extraction. Cells were disrupted by grinding the pellet using mortar and pestle and liquid nitrogen. A 5 ml volume of heated (65°C) phenol-chloroform and 5 ml of extraction buffer (10 mM EDTA, 1% sodium dodecyl sulphate (SDS), 2% 2-mercaptoethaol and 200 mM sodium acetate, pH 5) was added to the ground biomass. The RNA was precipitated by addition of 1/3 volume 8M lithium chloride (LiCl) enriched with 1% 2-mercaptoethanol. The RNA pellet was washed with 2M LiCl and twice with 70% ethanol. After evaporation of the last residues of ethanol, the pellet was resuspended in RNase free H_2O.

2.2.9 RNA sequencing and Transcriptome assembly

RNA-seq library preparation and deep sequencing on Illumina HiSeq2500 instruments were carried out at BaseClear (Leiden, The Netherlands). The Illumina TruSeq RNA sample preparation protocol was used to prepare libraries with a median fragment insert size of 230 bp. For all samples, 51 bp paired-end sequencing runs were carried out, data was delivered in Illumina format 1.8 and filtered for reads that did not pass the Illumina chastity filter, reads that aligned to the phiX control genome and reads that contained Illumina TruSeq adapters. To maximize the diversity and completeness of the *N. oleoabundans de novo* assembled transcripts, the data from 16 transcriptomes were combined yielding a total of 496,158,724 reads and assembled with IDBA-UD v1.1.0 (Peng et al., 2012). QUAST v2.3 (Gurevich et al., 2013) was used to estimate the quality of the assembly. Coding sequences (CDS) were extracted using QUAST and translated into protein sequences for functional annotation. The sequence data can be found at ArrayExpress from EBI, with the accession number E-MTAB-3746 .

2.2.10 Transcriptome Annotation

The protein sequences were annotated by aligning them against different databases by using DELTA-BLAST 2.2.29+ (default parameters, E-value < 0.001) (Boratyn et al., 2012) and by using InterproScan 5 (default parameters) for domain search analysis. Blast2GO V.2.7 (Conesa and Götz, 2008) was used as the central tool to combine both analysis methods to assign GO terms to the protein sequences and to retrieve EC numbers. For DELTA-BLAST the following databases were sequentially used: SwissProt, Chlorophyceae branch from SwissProt, Viridiplantae and Cyanobacteria branch from NCBI, Uniprot (SwissProt + Trembl) filtered for proteins with an annotated enzymatic reaction.

2.2.11 Expression analysis

Read abundance estimations were done using the RSEM script from TrinityRNAseq (Grabherr et al., 2011) with the default settings. Reads from each experimental condi-

tion were mapped onto the set of coding sequences generated with QUAST from the assembled transcriptome. Data was normalized taking into account the library sizes using Trimmed Mean of M-values (Robinson et al., 2010). They were further normalized by the CDS length to compute Fragments Per Kilo base of exon per Million fragments mapped (FPKM) using TrinityRNAseq TMM normalization script. CDS with FPKM values lower than 10 in all conditions were discarded. Finally, FPKM values corresponding to CDS annotated to the same enzyme or transporter present in the model were added up to provide a single expression value for the reactions in the model.

2.3 Results and Discussion

2.3.1 Biomass composition

N. oleoabundans was cultivated under four different conditions to assess the gene expression during growth in fresh water (A and B) and salt water (C and D), and for both conditions with nitrogen (A and C) and without nitrogen (B and D), to induce lipid accumulation (Figure 2.1A). Samples were taken at a time point when triacylglycerol (TAG) accumulation was induced but cells were not so stressed that apoptotic gene expression dominated the transcriptional profile (Figure 2.1B). In figure 2.2 (A-D), oil body formation in the *N. oleoabundans* cells is shown in the four different growth conditions. Compared to salt water adapted cells, cells grown in fresh water are much smaller, with diameters of approximately 4 and 8 µm respectively. The red fluorescence represents the autofluorescence of the chlorophylls and the neutral lipid bodies that are stained by BODIPY have a yellow fluorescence signal. Under nitrogen depleted conditions, there is significantly more BODIPY fluorescence indicating accumulation of neutral lipids that mainly consist of TAG molecules. Not all cells show the same neutral lipid content under nitrogen depleted conditions. This heterogeneity between cells has been observed previously in *N. oleoabundans* (de Winter et al., 2013; Klok et al., 2013a). This could be explained by the fact that, under constant light conditions, not every cell is in the same stage of the cell cycle when the TAG accumulation phase is induced. Individual differences in metabolic activity and internal nitrogen pool might determine whether cells start to produce TAG or not.

The total fatty acid (TFA) content increased as a consequence of the increase in TAG when the cells are exposed to nitrogen depleted conditions (Figure 2.2E). In the first 24 hours of nitrogen depletion, *N. oleoabundans* accumulates 12.1%±1.4 and 10.3%±0.8 of their dry weight as TAG molecules in fresh water and salt water conditions, respectively. The TFA and TAG content is therefore comparable for the salt water adapted cells compared to the fresh water culture, and the TAG fraction of the TFA content is the same under both conditions. In figure 2.2F (or Appendix Table 2.A1 relative to cell dry weight) the fatty acid profiles are shown for both TFA and TAG. No significant changes in fatty

acid composition are observed when comparing fresh water with salt water conditions, indicating that the salt resistance of *N. oleoabundans* is unlikely to be a consequence of modifications of the plasma membrane lipid composition. This is different for *Dunaliella salina,* which induces fatty acid elongation and expression of their related genes under saline stress (Azachi et al., 2002).

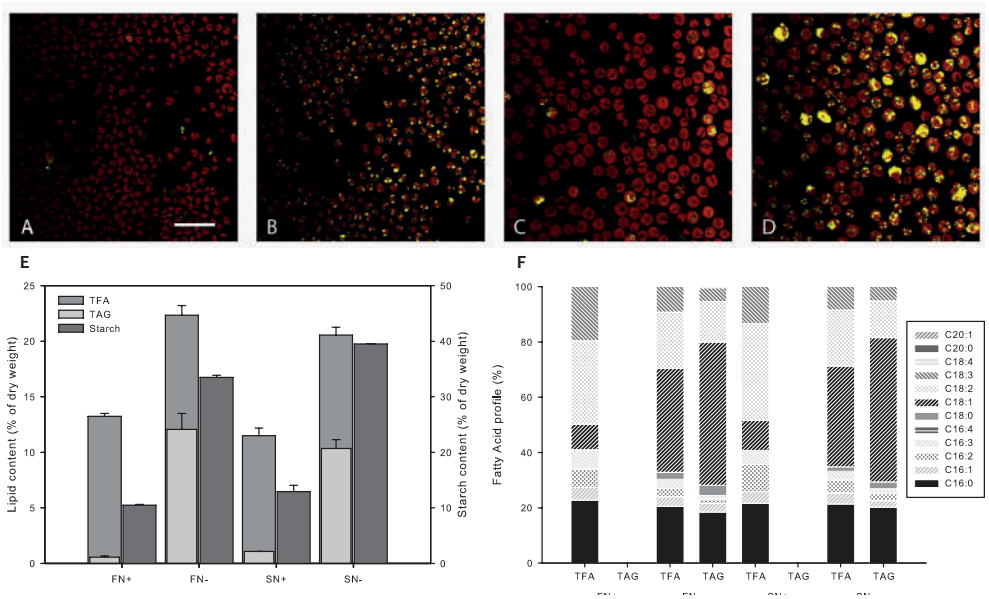

Figure 2.2 Biomass composition of *N. oleoabundans* in the four tested conditions. Upper panel: Confocal laser scanning microscope images of *N. oleoabundans* under four different cultivation conditions. (A) Nitrogen replete fresh water (FN+). (B) Nitrogen depleted fresh water conditions (FN-). (C) Nitrogen replete salt water conditions (SN+). (D) Nitrogen depleted salt water conditions (SN-). Chlorophyll autofluorescence is shown in red. BODIPY stain is shown in yellow. The bar represents 20 µm. Lower panel: (E) Lipid and starch content. TFA (dark gray), TAG (light gray), and starch (white) 24 hours after medium replacement. Error bars indicate the distance to the mean. (F) Fatty acid profile under the different conditions expressed as percentage of TFA or TAG.

RNAseq data analysis

De novo assembled transcripts were used to predict CDS that were further translated into putative protein sequences. In the 30489 assembled transcripts, QUAST detected 32136 putative protein sequences. Using the described method combining results from DELTA-BLAST and InterProScan through Blast2GO, we associated 8646 (28%) of the putative proteins to GO terms, counting 84836 associations with 5155 unique GO terms. From the putative proteins associated to GO terms, 3249 (38%) were additionally associated with an EC number, of which 834 were unique.

2.3.2 *Compatible solutes*

Compatible solutes are highly soluble low molecular weight molecules that can be accumulated to high concentrations without being toxic to the cell. They can protect cells against drought or saline stress by regenerating cellular osmotic homeostasis, relieving oxidative stress caused by Reactive Oxygen Species (ROS), and protecting membrane integrity and stabilization of enzymes or proteins (Bohnert and Jensen, 1996). Some examples of compatible solutes are, free amino acids, sugars, polyols and quaternary ammonium compounds (QAC).

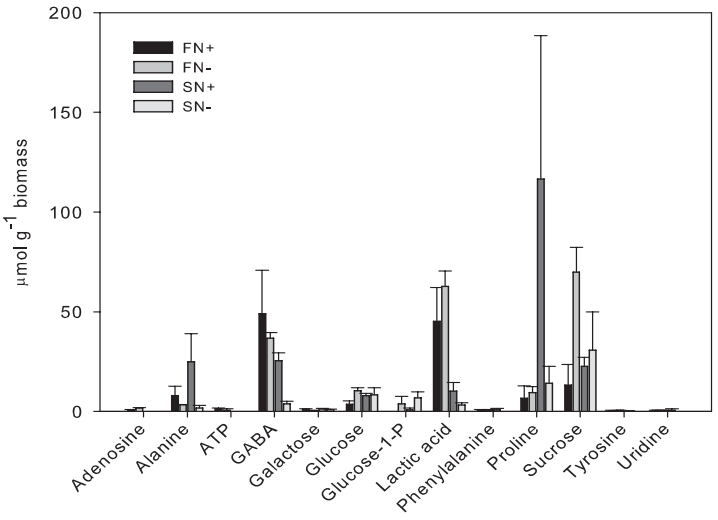

Figure 2.3 NMR spectroscopy showing metabolite concentrations under different conditions. Error bars represent distance to the mean of the duplicate samples.

2.3.2.1 Sugars

Several sugars are known to have a protective role in organisms experiencing different stress conditions. Examples of osmoprotectant sugars are trehalose and sucrose. The NMR spectroscopy analysis revealed that sucrose concentrations are increased under saline and nitrogen stress conditions (Figure 2.3). However, the intracellular level of sucrose under fresh water nitrogen depleted conditions is much higher than under salt water nitrogen depleted conditions, 69.9±12.3 µmol g^{-1} biomass and 30.7±19.1 µmol g^{-1} biomass respectively. In nitrogen depleted conditions sucrose levels increase 5.3 and 1.4 fold under fresh water and salt water conditions respectively. In most marine and freshwater cyanobacteria sucrose is synthesized from fructose-6-phosphate and UDP-glucose by the enzymes sucrose phosphate synthase (SucPS, EC:2.4.1.14) and sucrose-phosphate phosphatase (SucPP, EC:3.1.3.24) (Lunn, 2002). *Anabaena sp.* synthesizes sucrose in one step converting fructose and ADP or UDP glucose using sucrose synthase (SucST, EC:2.4.1.13) (Curatti et al., 2000). In *N. oleoabundans*, transcription of several genes in the

sucrose biosynthesis pathway are up-regulated under saline growth conditions. Transcripts are considered differentially expressed when the FPKM values are at least 1.5 fold lower or higher (log2 0.59) compared to the reference condition. In this pathway D-glucose-1P derived from glycolysis is converted into UDP glucose by the enzyme G1PUT which is marginally overexpressed under saline growth conditions. The conversion from UDP-glucose to sucrose is catalyzed by the enzyme sucrose synthase (SucST) and is strongly overexpressed under nitrogen depleted conditions (Figure 2.4).

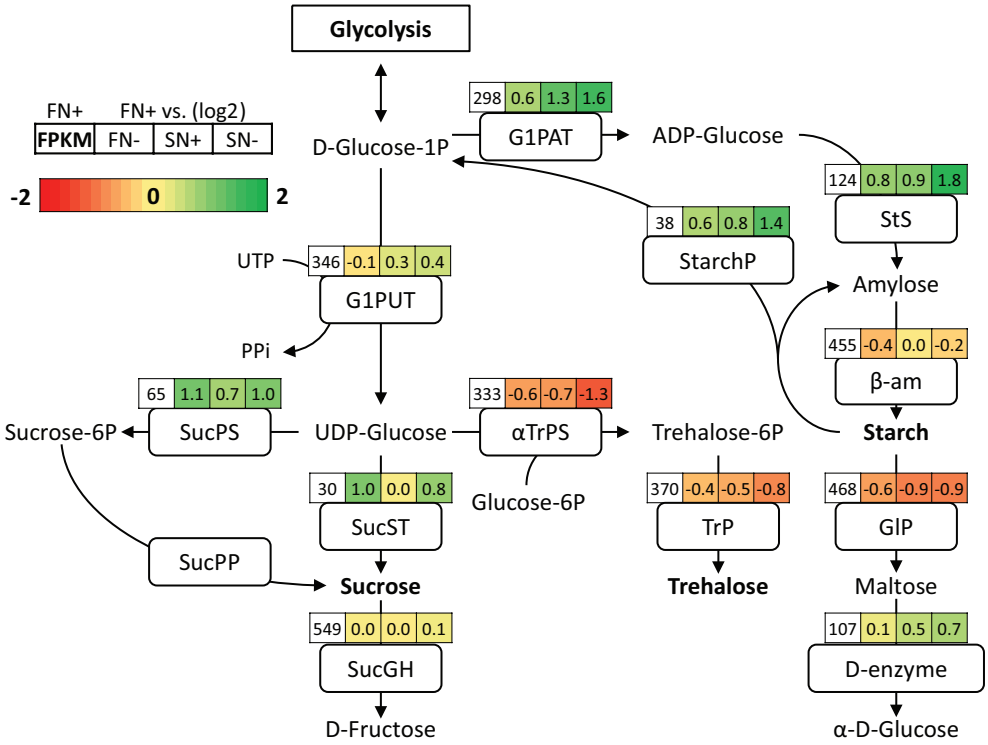

Figure 2.4 Biosynthesis pathway of sucrose and starch. The values that are shown in the following figures refer to the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) for each condition. The white left-most box represents the FPKM value of the respective gene in the fresh water nitrogen replete reference condition. This is followed by three colored boxes that represent the log2 fold change of the other conditions compared to the reference condition. The order of the remaining three boxes are from left to right, FN-, SN+, SN- respectively. Abbreviations: G1PUT: glucose-1-phosphate uridylyltransferase; UTP: Uridine triphosphate. SucPS: sucrose-phosphate synthase. SucST: sucrose synthase. G1PAT: glucose-1-phosphate adenylyltransferase. αTrPS: alpha,alpha-trehalose-phosphate synthase. αTrS: alpha,alpha-trehalose synthase. TrP: trehalose-phosphatase. StS:starch synthase. GlBE: glycogen branching enzyme. SucPP: sucrose-phosphate phosphatase. SucGH: sucrose glucohydrolase. β-am: β-amylase. D-enzyme: 4-alpha-glucanotransferase. StarchP: Starch phosphorylase.

The first step in the pathway that goes from UDP-glucose to sucrose via sucrose-6P catalyzed by SucPS is up-regulated under both salt water and nitrogen stressed cells. The second step catalyzed by sucrose–phosphate phosphatase (SucPP) is absent from the annotation. Sucrose can be degraded by sucrose glycohydrolase (SucGH) into D-fructose. Band et al. found that the main soluble carbohydrate that is accumulated in *N oleoabundans* experiencing a salt osmotic up-shock is sucrose (Band et al., 1992). Based on the sucrose levels measured and the expression levels of the genes involved, sucrose might be functioning as quick response to saline stress (Band et al., 1992), but does not seem to be responsible for salt resistance in long term salt adapted cells. Sucrose accumulation seems to be more of an overflow mechanism that results from nitrogen starvation.

Another sugar that is often found to be involved in salt resistance is trehalose. In high-temperature stressed yeast cells, trehalose concentrations are increased to protect enzymes from the elevated temperatures (Singer and Lindquist, 1998). Trehalose is found in different microalgal species under saline stress conditions (Bremauntz et al., 2011; Page-Sharp et al., 1999), but does not seem to be an osmoprotectant in *N. oleoabundans,* since key enzymes in this pathway are not transcriptionally up-regulated under saline conditions (Figure 2.4). This observation does however not completely exclude trehalose as a candidate for osmoprotection, because trehalose concentration was not analyzed directly and it could be that the flux toward this compound is not controlled at transcript level but on a metabolic level.

2.3.2.2 Proline

Proline is a proteinogenic amino acid that has strong conformational rigidity and is involved in primary metabolism. In bacteria proline levels are known to be related to the osmotic stress response (Csonka et al., 1988). In plants proline can function as a chemical chaperone in cell metabolism preventing protein aggregation when cells are exposed to extreme conditions such as high temperatures, heavy metals or osmotic stress, so that enzymes can function normally (Sharma and Shanker Dubey, 2005). Proline is known to be able to take action as an active osmolyte for osmotic adjustment as well. Proline also has an antioxidant function by reducing the harmful effect of singlet oxygen and hydroxyl radicals on photosystem II (PSII) (Alia et al., 1997; Matysik et al., 2002; Signorelli et al., 2014). Another important function of proline is maintaining the redox balance preventing photoinhibition or loss of photosynthetic efficiency by keeping the NADPH:$NADP^+$ ratio low enabling electron flow between reaction centers in the photosystems (Hare and Cress, 1997). Proline can reduce the peroxidation of lipids in *Chlorella vulgaris* and *Chlamydomonas reinhardtii* cells exposed to heavy metals and increase the activity of pyrroline-5-carboxylate synthase (P5CS, EC:1.2.1.88) and, in parallel, increase the intracellular reactive nitrogen species NO level (Mallick, 2004; Mehta and Gaur, 1999; Zhang et al., 2008). When proline accumulation was enhanced in transgenic *C. reinhardtii* by

over expressing a P5CS gene from mothbean, the levels of free radicals were reduced in reaction to the toxic metal cadmium (Siripornadulsil et al., 2002).

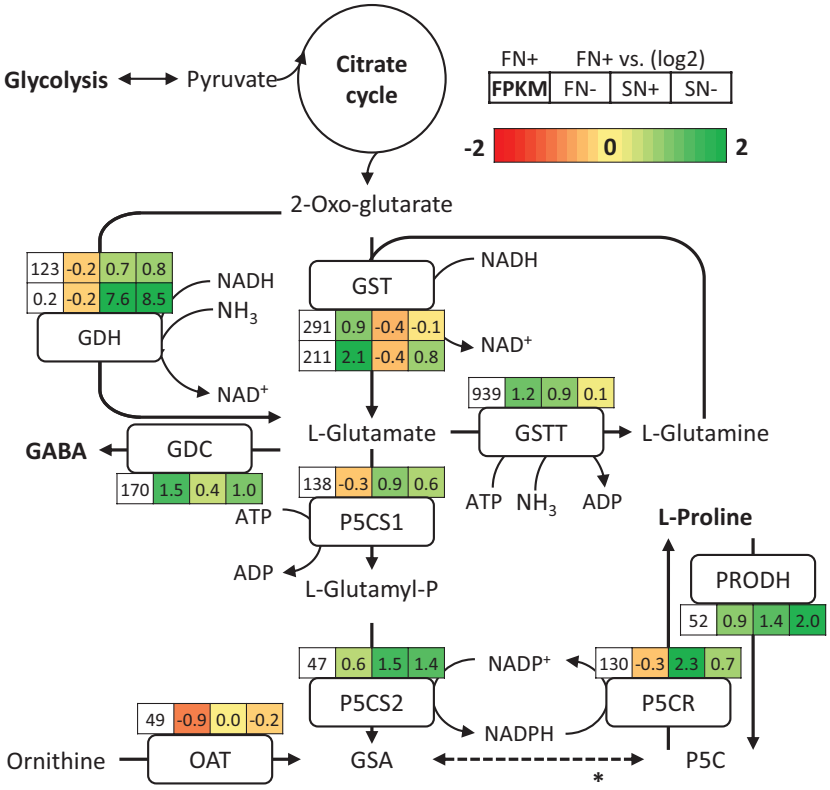

Figure 2.5 Proline and GABA biosynthesis pathway. For the figure legend refer to figure 2.4. Abbreviations: GSA: glutamate-semialdehyde. P5C: L-1-Pyrroline-5-carboxylate. GABA: 4-amino-butanoate. P5CR: P5C reductase. P5CS1: glutamate-5-kinase. P5CS2: GSA dehydrogenase. GDH: glutamate dehydrogenase (EC:1.4.1.3/4). GST: glutamate synthase (EC:1.4.1.13/14). GSTT: glutamine synthetase. GDC: glutamate decarboxylase. OAT: ornithine aminotransferase. PRODH: Proline dehydrogenase. *This reaction occurs non-enzymatically

The proline content was studied for all four conditions by NMR spectroscopy. The results are shown in figure 2.3. Under normal growth conditions there is around 30.7±6.3 µmol proline per gram of biomass. When cells are exposed to saline conditions the proline level is increased 7.5 times to 234.7±131.4 µmol g^{-1}. Saline conditions in combination with nitrogen stress resulted in a 2.5 times increase in proline concentration from 13.6±0.6 to 33.6±5.3 µmol g^{-1} for FN- and SN- respectively. Compared to fresh water conditions, under saline conditions the internal proline pool is significantly higher, suggesting that proline is the most likely compound involved in the saline resistance of *N. oleoabundans*.

In order to synthesize proline, in many organisms including plants and microalgae, inorganic nitrogen is first reduced to ammonium before it is used in the biosynthesis of several nitrogen containing metabolites such as amino acids (Krell et al., 2007; Rochaix and Merchant, 1998; Varshney and Koebner, 2010). Ammonium is assimilated into glutamate or glutamine, which functions as the precursor and donor for the amino acids proline and arginine. In higher plants proline is synthesized in the cytosol mainly from reduction of glutamate to glutamate-semialdehyde (GSA) by the bifunctional enzyme P5CS (Hong et al., 2000; Hu et al., 1992). In some organisms this enzymatic step is carried out by two separate enzymes, glutamate-5-kinase (P5CS1, EC:2.7.2.11) and glutamate-5-semialdehyde dehydrogenase (P5CS2, EC:1.2.1.41). Based on the transcriptome data this is also the case in *N. oleoabundans* (Figure 2.5) and based on the genome of the model species *C. reinhardtii* (Merchant et al., 2007). Unlike plants, microalgae and other microorganisms are not able to directly convert glutamate into GSA and that can be problematic, since the intermediate L-glutamyl 5-phosphate is rapidly cyclized into 5-oxoproline and phosphate. This issue is resolved by the formation of a complex of P5CS1 and P5CS2 to prevent the early cyclization of the labile intermediate L-glutamyl 5-phosphate in bacteria (Gamper and Moses, 1974), and a similar mechanism is believed to exist in microalgae (Merchant et al., 2007). Subsequently GSA is spontaneously converted to pyrroline-5-carboxylate (P5C) and further reduced by the enzyme P5C reductase (P5CR, EC:1.5.1.2) to proline. These final two steps are found in all organisms studied. Another way to accumulate proline is by converting ornithine to GSA and P5C by the enzyme ornithine-delta-aminotransferase (OAT, EC:2.6.1.13) (Figure 2.5).

In *N. oleoabundans,* many genes upstream of proline starting from the tricarboxylic acid (TCA) cycle are up-regulated under saline growth conditions. Based on the transcript levels it appears that the major route from the TCA cycle intermediate 2-oxo-glutarate towards L-glutamate is catalyzed via the enzyme glutamate dehydrogenase (GDH). There are two enzymes known that can catalyze this reaction: EC:1.4.1.3 and EC:1.4.1.4 using different co-factors. Both enzymes are up-regulated under saline growth conditions, log2 0.7 and log2 7.6 under nitrogen replete and log2 0.8 and log2 8.5 times in nitrogen depleted conditions (). The enzyme complex P5CS1 and P5CS2 converts L-glutamate into L-glutamyl-P and GSA respectively. Both enzymes are strongly up-regulated under saline growth conditions. For the P5CS1 gene this is log2 0.9 times and log2 0.6 times for nitrogen replete and depleted saline growth conditions respectively. For the P5CS2 enzyme, the up-regulation under the these conditions is log2 1.5 and log2 1.4 respectively. The alternative way to produce GSA by OAT from ornithine seems to be less important and only active under nitrogen replete conditions and with slightly higher expression under salt water conditions (Figure 2.5). In contrast, the diatom *Fragilariopsis cylindrus* is primarily using the ornithine pathway to generate proline as a response to salt stress (Krell et al., 2007). The final conversion from P5C to proline is markedly up-regulated under sa-

line nitrogen replete conditions, log2 2.3. Under nitrogen depleted salt water conditions the P5CR gene is also up-regulated but less, log2 0.7 fold compared to the fresh water nitrogen replete conditions.

The proline content in nitrogen starved cells is lower than in nitrogen replete grown cultures. The catabolism of proline is done by proline dehydrogenase (PRODH, EC:1.5.99.8) and was enhanced most strongly under nitrogen depleted conditions by log2 0.9 and log2 2.0 for fresh water and salt water growth conditions respectively. Under nitrogen depleted conditions PRODH is up-regulated to enable the recycling of nitrogen to supply nitrogen to other nitrogen requiring processes. Under salt water replete conditions, the PRODH gene expression is also strongly up-regulated, log2 1.4. This can be explained by the fact that the proline content is tightly regulated by PRODH under salt water conditions.

To conclude, since proline levels are increased in salt water adapted cells and all genes involved in the proline biosynthesis are up-regulated, proline is most likely to be the primary mechanism in the saline resistance of *N. oleoabundans*. Furthermore, under nitrogen depleted conditions, PRODH is up-regulated to enable the recycling of nitrogen to use the nitrogen molecules from proline in other nitrogen requiring processes in the cell.

2.3.2.3 Other potential osmoregulatory mechanisms

Glycine betaine

The use of glycine betaine (GB) as an osmoprotectant is not widespread throughout the plant kingdom. Rice, tobacco, Arabidopsis, and mustard do not produce GB naturally. Transgenic plants that are able to accumulate GB, have their salt resistance increased (Rhodes and Hanson, 1993), indicating that GB accumulation can be an effective solution to counter osmotic stress resulting from saline environments. The freshwater cyanobacterium *Synechococcus sp.* is lacking the pathway to accumulate GB from choline. Transgenic strains that are complemented with the necessary genes from *E. coli* accumulated GB and had a higher resistance to salt water than the wild type when in combination with exogenous supply of choline, partly owing to stabilization of photosystem II (Nomura et al., 1995). GB is synthesized by converting choline into betaine aldehyde. In plants this step occurs in the chloroplast and is catalyzed by the enzyme choline monooxygenase (CholMO, EC:1.14.15.7) which is not found in *N. oleoabundans*. In many bacteria and mammals, the membrane bound enzyme choline dehydrogenase (CholDH, EC:1.1.99.1) or soluble enzyme choline oxidase (CholOx, EC:1.1.3.17) is responsible for this step. The latter is not found *in N. oleoabundans*. CholDH is present in *N. oleoabundans* and its expression is strongly down-regulated under nitrogen depleted conditions (Appendix Table 2.A2). This can be explained by the fact that the lack of nitrogen is resulting in down-regulation of the betaine pathway to use the nitrogen elsewhere. The second step from betaine

aldehyde to betaine is catalyzed by betaine-aldehyde dehydrogenase (BADG, EC:1.2.1.8) and seems to be universal in plants bacteria and animals. This enzyme was not annotated for *N. oleoabundans*. An alternative pathway is known in bacteria using direct N-methylation of glycine via sarcosine. It is possible that betaine plays a role in osmoregulation in *N. oleoabundans*, but there is no strong evidence based on gene expression and the lack of direct betaine measurements.

Dimethylsulfoniopropionate (DMSP)

DMSP is a sulfonium compound that is known to be accumulated in several marine microalgae and some plants (Gage et al., 1997; Hanson et al., 1996). DMSP functions as a compatible solute similar to betaine-like osmolytes, but has the advantage that the compound does not contain nitrogen and could therefore be used as an osmoprotectant under nitrogen limiting conditions. The biosynthesis of DMSP is different in microalgae and plants. Higher plants produce DMSP from S-methylmethionine while in the microalgae *Enteromorpha intestinalis* it was discovered that methionine is the precursor compound (Gage et al., 1997).

Methionine is transaminated to 4-methylhio-2-oxobutyrate (MTOB) and reduced to 4-methylhio-2-hydroxybutyrate (MTHB). MTHB is S-methylated into 4-dimethylsulfonio-2-oxobutyrate (DMSHB) which is oxidatively decarboxylated to DMSP (Summers et al., 1998). The first step from methionine to MTOB can be catalyzed by two aminotransferases (EC:2.6.1.5 EC:2.6.1.57). Both enzymes are annotated in *N. oleoabundans* and transcriptome data suggest that they are up-regulated in salt water growth conditions. The first aminotransferase is up-regulated log2 0.5 and log2 0.1 under salt water nitrogen replete conditions and the second aminotransferase is up-regulated log2 0.9 and log2 0.6 under salt water nitrogen replete conditions (Appendix Table 2.A2). Evidence for the existence of other downstream enzymes could not be extracted from the transcriptome data. There is still not much known about the exact enzymes involved in the DMSP biosynthesis pathway, making it hard to study transcript levels of genes involved in this pathway. The nitrogen rich amino acids will be reshuffled and recycled upon nitrogen starvation by transamination reactions. These transamination reactions can promote DMSP synthesis, since the first step in DMSP synthesis is the transamination of methionine to MTOB (Wolfe et al., 1997). It is difficult to draw conclusion on the role of DMSP as potential osmoprotectant in *N. oleoabundans*, since DMSP was not measured in the NMR analysis.

γ-Aminobutyrate

In *N. oleoabundans,* the γ-Aminobutyrate (GABA) content seems to decline in proportion to the amount of stress experienced (Figure 2.3). The highest GABA concentration is found in nitrogen replete fresh water conditions and the lowest content is found in salt

water nitrogen depleted conditions. This is not in correspondence with previous studies that investigated plant species, where GABA is found to be a protective osmolyte and can be accumulated in high amounts. Based on the NMR analysis, GABA does not seem to be of importance in the saline resistance of *N. oleoabundans*. The enzyme responsible for the conversion of L-Glutamate to GABA, glutamate decarboxylase (GDC, EC:4.1.1.15), is differentially expressed, but there is more expression of this gene under nitrogen depleted conditions than under saline conditions (Figure 2.5).

ATPases

Transmembrane adenosinetriphosphatase (ATPases) are a class of enzymes (EC:3.6.1.3) that use the energy released from dephosphorylation of ATP into ADP and free phosphate to transport ions across a membrane against a concentration gradient, thus enabling reactions that would not occur spontaneously. These transmembrane pumps are able to import nutrients into the cell or exclude toxic compounds from the cell. The enzyme group ATPases is a diverse generalized name for active transmembrane pumps. In this study 52 ATPases were found to be differentially expressed under the tested conditions. Around half of these transcripts seem to be correlated to salt water stress ranging from log2 8.3 increase to log2 -9.2 decrease (Appendix Table 2.A3). An example of an ATPases known to be involved in osmotic regulation is the Na^+-pump ATPase in *Dunaliella maritima* located in the plasma membrane (Popova et al., 2005). In *N. oleoabundans* the Na^+/K^+ pump (EC:3.6.3.9) is strongly up-regulated under salt conditions and even more in combination with nitrogen limitation (Appendix Table 2.A3). This pump is normally exclusively found in animal cells and not in plant or microalgal cells. It is likely that this transcript was wrongly annotated and is actually a different ATPase with close sequence similarity to the animal like Na^+/K^+ pump. The H^+ exporting ATPase (EC:3.6.3.6) was found to be responsible for proton-pumping pyrophosphate into polyphosphate bodies in *C. reinhardtii* (Ruiz et al., 2001). This ATPase is up-regulated in *N. oleoabundans* under salt and nitrogen stress, and enables the cell to decrease the acidity of the cytoplasm caused by stress, by pumping H^+ ions and cations such as Ca^{2+} to vacuoles. Other ions that need to be actively excluded from the cell under salt stress are Mg^{2+} and Ca^{2+}. These cations are actively transported from the cell to the surrounding environment by the Mg^{2+}-translocating ATPase (EC:3.6.3.2) and Ca^{2+} ATPase (EC: 3.6.3.8), respectively. Both enzymes are not found to be up-regulated under any of the conditions tested in *N. oleoabundans*.

Polyol pathway

A polyol is an alcohol containing several hydroxyl groups and polyols are found in many organisms. Examples of polyols are glycerol, mannitol, and sorbitol. The water-like hydroxyl groups in the polyols can prevent dehydration of molecules under salt water (Galinski and Truper, 1994). Many plant and fungal species use this trick to sustain metabolic homeostasis and to overcome the negative effects of osmotic stress (Williamson et

al., 2002). Some microalgae accumulate polyols in response to osmotic stress. Different *Dunaliella* species are known to be resistant to saline conditions because of the accumulation of glycerol (Borowitzka and Brown, 1974; Ginzburg, 1987). Band et al. found that glycerol levels are not increased in salt shocked *N. oleoabundans* cultures (Band et al., 1992). Also in this study, there is no evidence for assigning the saline resistance of *N. oleoabundans* to glycerol or any other polyol. There is no evidence for polyol accumulation based on the gene expression of genes involved in the pathways for compounds such as sorbitol, mannitol, glycerol or any other polyol.

2.3.3 Oxidative stress

Under stress conditions ROS can be formed which need to be removed to prevent damage to the photosystems and other cellular equipment (Smirnoff, 1993). The oxidative stress can be relieved by antioxidant enzymes, such as superoxide dismutase (SOD; EC:1.15.1.1), which is highly overexpressed under saline conditions log2 1.1 and log2 1.5 fold in nitrogen replete and depleted conditions respectively (Figure 2.6) and catalase (CAT, EC 1.11.1.6) which is present under nitrogen depleted conditions and strongly down regulated under nitrogen replete saline conditions. Glutathione (GSH) is a pivotal compound for many plant species and microalgae to scavenge ROS, such as superoxide hydrogen peroxide and lipid hydroperoxides, which can be accumulated under environmental and oxidative stress (Zhang et al., 2013). In addition to direct scavenging of ROS, GSH can also function as the reductant in the glutathione-ascorbate cycle which can alleviate ROS build up and provide protein protection. The tripeptide glutathione (GSH) is synthesized from glutamate and cysteine into γ-glutamylcysteine by γ-glutamylcysteine synthetase (γ-GCSTT EC:6.3.2.2) at the expense of one ATP. In the next step another ATP molecule is needed to convert γ-glutamylcysteine and glycine into glutathione, by glutathione synthetase (GSHSTT EC:6.3.2.3) (Figure 2.6).

Another way to relieve the pressure of these harmful radicals is by oxidizing GSH in a metabolic cycle that is known as the ascorbate-glutathione cycle. First superoxide is converted into hydrogen peroxide by the enzyme superoxide dismutase. The hydrogen peroxide is a toxic compound as well and can be converted to H_2O by oxidation of ascorbate to monodehydroascorbate (MDHA) by ascorbate peroxidase (APX EC:1.11.1.11). This enzyme could not be annotated in *N. oleoabundans* from the mRNA sequences. MDHA needs to be converted back to ascorbate, which can be done in two ways. Enzymatically by monodehydroascorbate reductase (MDHAR EC:1.6.5.4) or non-enzymatically by spontaneous disproportionation of two MDHA molecules resulting in ascorbate and dehydroascorbate (DHA) (Smirnoff, 2000). DHA will subsequently be converted back to ascorbate by dehydroascorbate reductase (DHAR EC:1.8.5.1) coupling two GSH molecules into glutathione disulfide (GSSG). Glutathione reductase (GSHR EC:1.8.1.7) can catalyze the reduction of GSSG back to two GSH molecules.

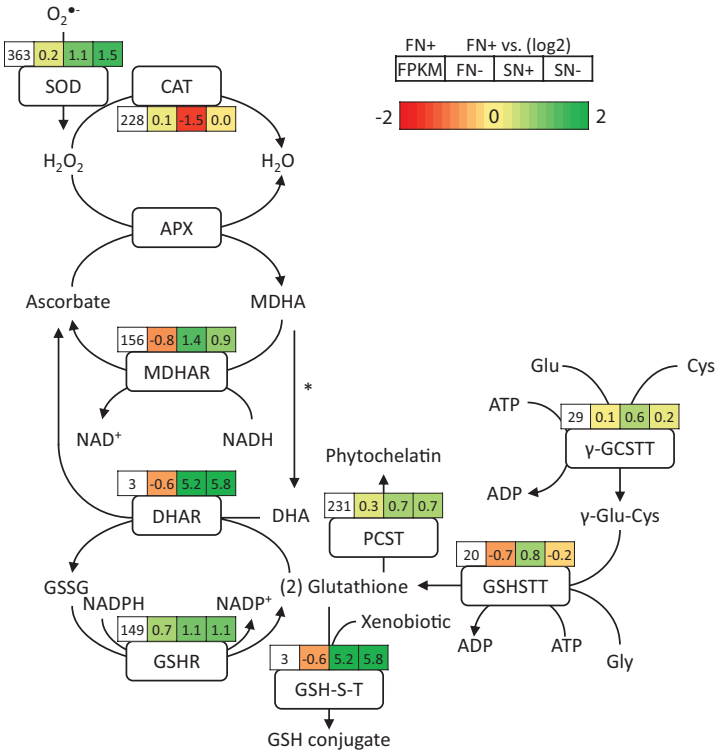

Figure 2.6 Glutathione biosynthesis pathway and glutathione-ascorbate cycle. For the figure legend refer to figure 2.4. Abbreviations: SOD Superoxide dismutase; CAT Catalase; APX Ascorbate peroxidase (not annotated); MDHA monodehydroascorbate; MDHAR monodehydroascorbate reductase; DHA dehydroascorbate; DHAR dehydroascorbate reductase; GSH (reduced) Glutathione; GSSG oxidised glutathione; GSHR Glutathione reductase; Glu Glutamate; Cys Cysteine; Gly Glyceine; γ-GCSTT γ-glutamylcystein Synthetase; γ-Glu-Cys γ-glutamylcystein; GSHSTT Glutathione Synthetase; GSH-S-T glutathione S-transferase; PCST Phytochelatin synthase. * This reaction can occur enzymatically or non-enzymatically.

The efficiency of GSH is dependent on the GSH concentration in the cell which in turn depends on the activity of the GSH reductase enzyme which determines the ratio between GSH and its oxidized form GSSG. All genes involved in the ascorbate-GSH cycle were up-regulated under salt water growth conditions, including the biosynthesis of GSH (Figure 2.6). The MDHA reductase gene is log2 1.4 fold increased under saline replete conditions and log2 0.9 fold increased under nitrogen depleted conditions. DHA reductase was strongly up-regulated under salt water conditions to regenerate the ascorbate from DHA. DHAR was log2 5.2 times and log2 5.8 up-regulated under nitrogen replete and depleted salt water growth conditions respectively. To recycle the GSH molecules, GSHR is up-regulated under salt water nitrogen replete and depleted conditions as well, namely log2 1.1 and log2 1.1 times respectively.

Another function of GSH is the detoxification of xenobiotics, compounds that have no significant nutritional function in cell metabolism, but do affect cellular homeostasis in too high concentrations, this in contrast to compatible osmolytes that do not interfere with metabolism when present in high concentrations. The xenobiotic molecules can be conjugated to GSH, by GSH-S-transferase (GSHT, EC:2.5.1.18), and transported to vacuoles to be detoxified. Under saline conditions this enzyme is strongly up-regulated, log2 5.2 and log2 5.8 fold under nitrogen replete and depleted saline conditions respectively. This indicates the likeliness of GSH conjugate formation and exclusion to relieve xenobiotic pressure under saline conditions (Figure 2.6). It is known in plants that GSHT is induced under salt and drought stress to reduce the ROS in plants and GSHT could also be involved in reducing the harmful byproducts of oxidative stress such as lipid peroxidation (Marrs, 1996; Sappl et al., 2009).

A third protecting feature of GSH is the formation of phytochelatins (PC) by the enzyme phytochelatin synthase (PCST EC:2.3.2.15). PCs are oligomers of GSH and are able to detoxify heavy metals by chelation of toxic ions (Howe and Merchant, 1992; Hu et al., 2001). The PC-ion complexes can be compartmentalized in the vacuole of plants where they can do no further harm. PC has also been found to be involved in stress responses other than to heavy metals. In the cyanobacterium *Anabaena doliolum* PCs are produced in response to UV-B radiation (Bhargava et al., 2005). The cloning of the PC synthase gene of *Anabaena sp.* into *E. coli* increased the resistance of this bacterium to heat, metals, UV-B, salt, and herbicides (Chaurasia et al., 2008). Some plants have been shown to produce PCs in response to heat or salt stress (Seki et al., 2002; Zhang et al., 2005). The *N. oleoabundans* PCST gene is up-regulated in salt water medium under both nitrogen conditions (log2 0.7 fold), indicating that PCs are likely to be formed to protect *N. oleoabundans* against osmotic stress. Based on the general increase in expression of the genes involved in the ascorbate glutathione cycle, it is very likely that *N. oleoabundans* is using this cycle to alleviate the pressure of ROS that arise under saline growth conditions and that the GSH derived conjugates and phytochelatins GSH oligomers are likely to be involved as well. This mechanism is not used to alleviate the ROS pressure under nitrogen stress, at least not under the tested conditions.

2.3.4 Starch and triacylglycerol accumulation

2.3.4.1 Starch pathway

Starch is known to be transiently accumulated under nitrogen depleted conditions in the beginning of the stress phase (Breuer et al., 2014; de Jaeger et al., 2014; de Winter et al., 2013). In this study the sample time was 24 hours after nitrogen stress induction and it is expected that the genes involved in starch biosynthesis would be up-regulated in this early phase of nitrogen stress. The starch levels under the four different conditions are shown to be strongly increased in the first 24 hours after nitrogen starvation. Under

fresh water conditions, the starch content increased from 10.5%±0.1 to 33.5%±0.4 when switched to nitrogen depletion and from 12.9%±01.1 to 39.5%±0.0 under nitrogen depletion in salt water conditions (figure 2.2E). Interestingly, salt water adapted cells had a 1.2 times higher starch content compared to the fresh water adapted cells under both nitrogen replete and depleted conditions.

The genes encoding glucose-1-phosphate adenylyltransferase (G1PAT, EC:2.7.7.27) and starch synthase (StS, EC:2.4.1.21) are strongly up-regulated under nitrogen depleted conditions, but seem to be affected by salt water as well (Figure 2.4). The G1PAT gene is log2 0.6 times up-regulated in the freshwater nitrogen stressed culture, while in salt water adapted cultures, this gene is log2 1.3 and log2 1.6 times up-regulated under nitrogen replete and depleted conditions respectively. Starch accumulation upon nitrogen starvation seems to be facilitated by transcriptional up-regulation of starch biosynthesis genes with the exception of the final step catalyzed by glycogen branching enzyme (GlBE, EC:2.4.1.18).

Starch catabolism can be facilitated by different enzymes. β-amylase (β-am, EC:3.2.1.2) degrades starch by removing maltose units from the non-reducing ends of the chains. β-am was shown to be down-regulated under salt water and nitrogen depleted conditions (Figure 2.4). Unexpectedly, starch phosphorylase (StarchP, EC:2.4.1.1) and α-amylase (α-am, EC:3.2.1.1), two other starch degrading enzymes, are up-regulated under nitrogen depleted conditions when there is accumulation of starch (Appendix Table 2.A2). A possible explanation could be that the starch turnover is very high and that there is a balance between starch biosynthesis and starch catabolism. When starch is accumulated the ratio between starch biosynthesis and catabolism shifts more towards starch biosynthesis. Another reason could be that under nitrogen limiting conditions starch is degraded to rearrange the carbon stored in starch towards TAG. Salt stress does seem to increase the starch content, which is possibly as a result of reduced growth such that more energy needs to be channeled from the photosystems and is fixed in starch (Jia et al., 2015).

2.3.4.2 Triacylglycerol pathway

When oleaginous microalgae are exposed to unfavorable growth conditions such as nitrogen depleted conditions, cells start to accumulate triacylglycerol (TAG). These neutral glycerolipids are composed of a glycerol backbone with three fatty acid molecules attached. The fatty acid biosynthesis takes place in the chloroplasts and starts downstream of the glycolysis with the conversion of acteyl-CoA to malonyl-CoA by the heteromeric enzyme complex acetyl-CoA carboxylase (ACCase). The acetyl-CoA pool can be supplied in two different ways. The first way is via pyruvate and the pyruvate dehydrogenase complex, in which the first two steps are up-regulated under nitrogen depleted

conditions in *N. oleoabundans* (EC:1.2.4.1 and EC:1.8.1.4, see Figure 2.7). Another way is via the TCA cycle in which citrate can be converted into acetyl-CoA and oxaloacetate by ATP citrate lyase (EC:2.3.3.8) (Rangasamy and Ratledge, 2000). The *N. oleoabundans* ATP citrate lyase is up-regulated under nitrogen depleted conditions and it is likely that this pathway is involved in supplying part of the acetyl-CoA.

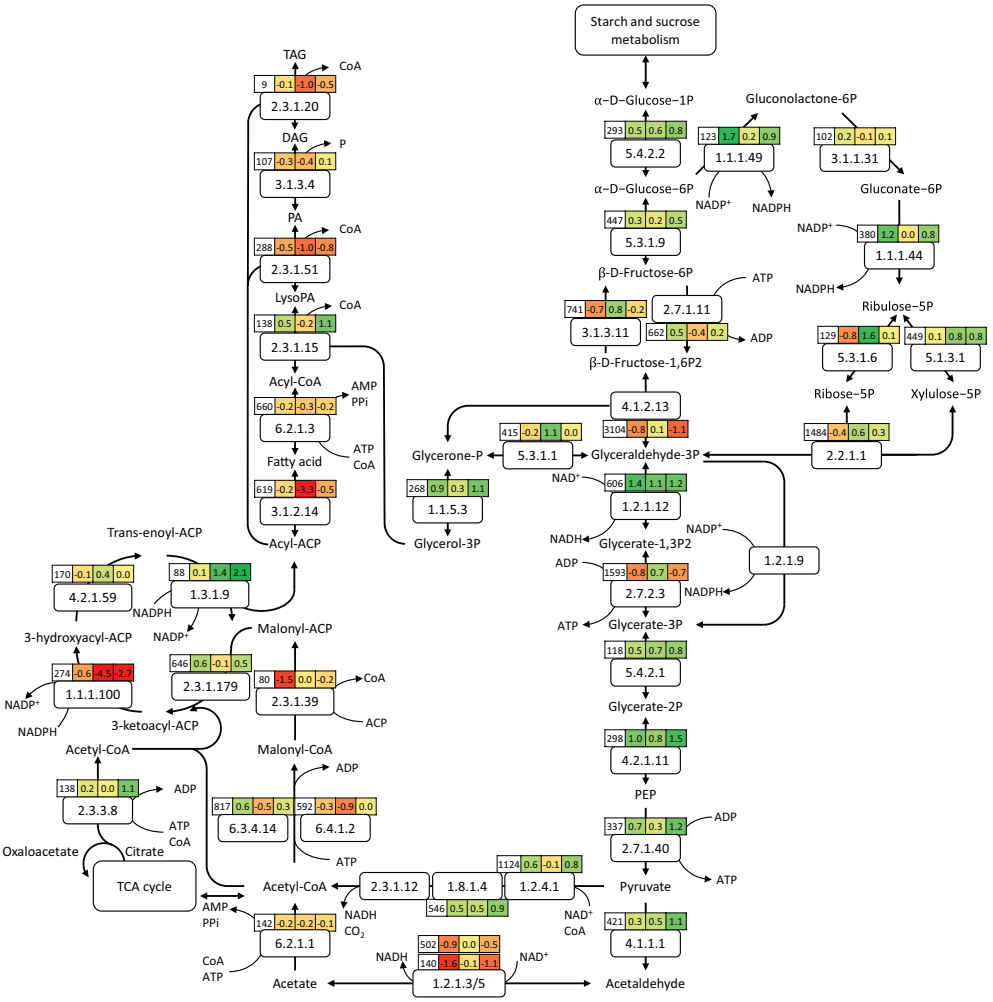

Figure 2.7 Carbon metabolism in N. oleoabundans. For the figure legend refer to figure 2.4. Codes in the white boxes represent the corresponding EC numbers. Abbreviations: PEP phosphoenol pyruvate; LysoPA Lysophosphatidic acid; PA Phosphatidic acid; DAG 1,2-Diacylglycerol; TAG Triacylglycerol. EC:2.3.1.12 could not be annotated from the transcriptome.

ACCase catalyzes an important step in fatty acid biosynthesis and overexpression of this enzyme resulted in increased lipid contents in *Arabidopsis* (Roesler et al., 1997), but not necessarily in microalgae (Dunahay et al., 1996). Over expression of the ACCase complex

is challenging because of the multigene-encoded enzyme complex and post-translational modifications (Nikolau et al., 2003; Ohlrogge and Jaworski, 1997). Next, an acyl-carrier protein (ACP) is exchanged for the CoA moiety by malonyl-CoA:ACP transacylase giving rise to a malonyl-CoA molecule. Malonyl-CoA enters the heteromultimeric fatty acid synthesis (FAS) cycle where it is extended with two carbon atoms per repetitive cycle to usually 16 or 18 carbon long acyl-ACP groups.

In this study, two subunits of the enzyme complex ACCase could be identified, biotin carboxylase (EC: 6.3.4.14) and biotin carboxyl carrier (EC:6.4.1.2). The biotin carboxylase subunit is up-regulated under nitrogen depleted conditions, and the biotin carboxyl carrier does not seem to be strongly up-regulated under nitrogen depleted conditions (Figure 2.7). With the exception of the first and last step of the FAS cycle: beta-ketoacyl-ACP synthase II (EC:2.3.1.179) and enoyl-ACP reductase (EC:1.3.1.9) (Figure 2.7). Since the TAG content is strongly increased in the first 24 hours of nitrogen starvation (Figure 2.2), it was expected that transcription of the genes encoding the enzymes involved in the TAG synthesis pathway would be up-regulated under nitrogen depleted conditions. This was not the case with the exception of the glycerol 3-phosphate acyltransferase (EC:2.3.1.15) gene, which showed a log2 0.5 and log2 1.1 fold change under fresh water and salt water nitrogen depleted conditions respectively (Figure 2.7). This enzyme links glycolysis and the TAG synthesis pathway by attaching the first acyl-ACP molecule to sn-Glycerol 3-phosphate resulting in lysophosphatidic acid. Glycerol-3-phosphate can be formed from glycerol via glycerol kinase (EC:2.7.1.30) or from dihydroxyacetone phosphate via glycerol-3-phosphate dehydrogenase (EC:1.1.5.3). The latter seems to be the case in *N. oleoabundans* since transcription of the gene for this enzyme is strongly up-regulated under nitrogen depleted conditions. The overexpression of a yeast glycerol-3-phosphate dehydrogenase in rape seed, resulted in a 40% increase of the seed oil content, indicating that the available glycerol backbones are a limitation in TAG accumulation in rape seed (Vigeolas et al., 2007). To ensure sufficient supply of G3P, transcription of triose-phosphate isomerase (EC:5.3.1.1), the enzyme that converts glycerone-P (Dihydroxyacetone phosphate, DHAP) to glyceraldehyde-3-P, is reduced under nitrogen depleted conditions (Figure 2.7). Chen et al. described the increase of DHAP, G3P and glycerol in *Arabidopsis* when triose-phosphate isomerase was knocked out (Chen and Thelen, 2010). A similar regulatory mechanism was found in microalgae (Gao et al., 2014).

The last step from diacylglycerol (DAG) to triacylglycerol (TAG) is catalyzed by diacylglycerol O-acyltransferase (DGAT, EC:2.3.1.20). This enzyme is down regulated in *N. oleoabundans* under all tested conditions compared to the fresh water replete conditions. DGAT has been extensively studied since it is regarded as one of the most important rate limiting steps in TAG biosynthesis. Over expression of this gene or several subunits have resulted in very different outputs. In *Phaeodactylum tricornutum* there have been

very promising results, resulting in higher levels of TAG synthesis, but in the green alga *C. reinhardtii* there have not been consistent increases in TAG content in combination with DGAT over expression (Deng et al., 2012; Hsieh et al., 2012; La Russa et al., 2012; Niu et al., 2013). The fact that DGAT does not seem to be over expressed under nitrogen depleted conditions is surprising, since TAG accumulation is occurring in *N. oleoabundans* under these circumstances. In this study only one DGAT gene could be identified and it is known from other species that there are several genes that encode for DGAT enzymes and that they have very different expression profiles as a response to nitrogen deprivation (Jia et al., 2015; Li et al., 2014; Wang et al., 2014). More research needs to be done to understand and identify the different DGAT enzymes in *N. oleoabundans*.

As can be seen in figure 2.7, most of the genes involved in energy and carbon metabolism are up-regulated under nitrogen depleted conditions. Transcription of genes involved in glycolysis, the TCA cycle, and the pentose phosphate pathway (PPP) increased, also under salt conditions. This can be explained by the need for sufficient carbon precursors such as acetyl-CoA and ATP. Furthermore the PPP might be used to redirect the stored carbon in starch towards TAG biosynthesis as has been hypothesized before (Breuer et al., 2014). In the PPP, the transcripts for the NADPH generating enzymes are strongly up-regulated to generate the required NADPH that is needed for TAG biosynthesis and to scavenge ROS.

To maintain high levels of TAG molecules the rate of TAG catabolism should be low (Trentacoste et al., 2013). The gene triacylglycerol lipase (EC:3.1.1.3) is highly expressed under nitrogen replete fresh water conditions (FPKM: 1734). The expression is down regulated under nitrogen depleted and salt water conditions, log2 -0.3, log2 -0.9, and log2 -0.6 for fresh water nitrogen-depleted, salt water nitrogen replete and depleted conditions respectively (Appendix Table 2.A2).

2.4 Conclusion

To assess the metabolic and transcriptomic response to nitrogen and osmotic stress, *N. oleoabundans* was cultured under four different conditions. Transcriptome analysis suggested a number of potential compatible osmolytes dedicated to alleviate osmotic stress. Transcription of genes involved in proline biosynthesis are up-regulated under saline conditions, the most likely compatible solute in *N. oleoabundans* is proline. Proline is a known chemical chaperone, which can protect the photosynthetic apparatus and alleviate the damaging effects of ROS. Another potential osmoprotectant identified from the transcriptomic analysis in *N. oleoabundans* is glutathione ,and the pathways involving GSH such as the ascorbate-GSH cycle, GSH-conjugates and PC formation. All these pathways relieve oxidative stress and can neutralize toxic compounds such as xe-

nobiotics. To protect cells from oxidative injury caused by abiotic stresses, genes encoding antioxidant enzymes, such as superoxide dismutase are highly expressed under saline conditions. Unlike other microalgae, the plasma membrane lipid composition was not adjusted in salt water adapted cells. Transcriptome analysis suggests that under nitrogen limiting conditions, sucrose, starch, and TAG are accumulated and the genes in the respective pathways are up-regulated. For both fresh water and salt water adapted cultures under nitrogen depleted conditions the transcriptome levels were for carbon metabolism were similar. The glycerol 3-phosphate acyltransferase and glycerol-3-phosphate dehydrogenase genes were identified as a promising target for future research focusing on enhancing TAG production in microalgae, since it links the glycolysis to the TAG biosynthesis pathway. The results of this study can be used to develop strategies to enhance salt resistance aim other industrial relevant microalgal strains and plants. Hereby reducing the dependence on precious fresh water resources and arable land.

Acknowledgements

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Appendix

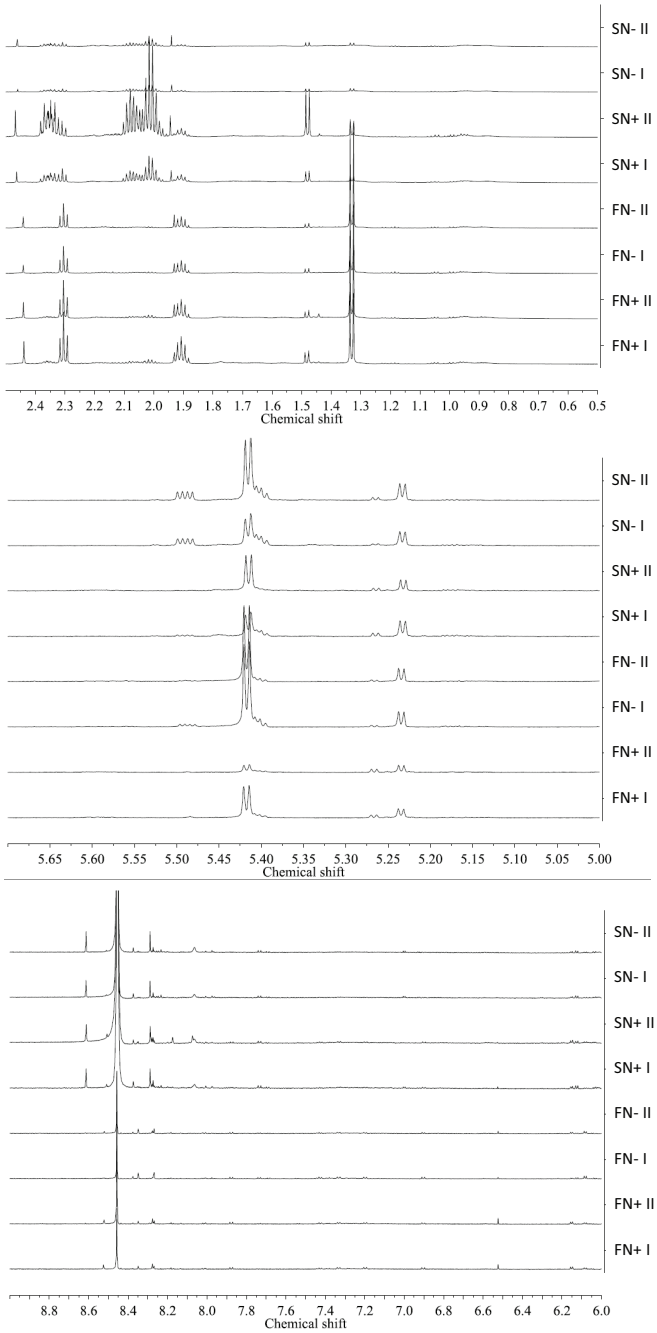

Figure 2.A1 NMR Quantitative variation in the 1H NMR spectra from all four conditions in duplicate. Spectra are derived from ethanol–water extracts of individual samples of FN+ fresh water replete, FN- Fresh water nitrogen depleted, SN+ Salt water replete, SN- salt water nitrogen depleted conditions The numbers I and II indicate duplicates.

Table 2.A1 Fatty acid composition of total fatty acid and triacylglycerol lipids expressed as fraction of dry cell weight. Values are the average of the biological duplicates.

	FN+		FN-		SN+		SN-	
	TFA	TAG	TFA	TAG	TFA	TAG	TFA	TAG
C16:0	2.7 ± 0.0	0.1 ± 0.0	4.2 ± 0.1	2.2 ± 0.2	2.3 ± 0.2	0.2 ± 0.0	4.0 ± 0.1	2.1 ± 0.1
C16:1	0.6 ± 0.0	0.0 ± 0.0	0.7 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.8 ± 0.0	0.2 ± 0.0
C16:2	0.8 ± 0.1	0.0 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	1.0 ± 0.1	0.0 ± 0.0	0.9 ± 0.0	0.3 ± 0.0
C16:3	0.8 ± 0.0	0.0 ± 0.0	0.7 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.6 ± 0.0	0.2 ± 0.0
C16:4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C18:0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.3 ± 0.0	0.2 ± 0.0
C18:1	1.1 ± 0.1	0.1 ± 0.1	7.8 ± 0.8	6.3 ± 0.9	1.2 ± 0.1	0.1 ± 0.1	6.9 ± 0.5	5.4 ± 0.5
C18:2	3.6 ± 0.2	0.1 ± 0.0	4.2 ± 0.1	1.8 ± 0.2	3.7 ± 0.2	0.3 ± 0.0	3.8 ± 0.1	1.4 ± 0.1
C18:3	2.4 ± 0.1	0.1 ± 0.0	1.9 ± 0.1	0.6 ± 0.0	1.4 ± 0.2	0.1 ± 0.0	1.6 ± 0.0	0.5 ± 0.0
C18:4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C20:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C20:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SUM	12.0 ± 0.2	0.5 ± 0.1	20.6 ± 0.8	12.0 ± 1.4	10.6 ± 0.7	1.1 ± 0.0	18.9 ± 0.7	10.3 ± 0.8

Table 2.A2 FPKM values and fold change for all transcripts discussed in this manuscript.

EC number	**FPKM**	**FPKM fold change (log2)**		
		FN+ vs FN-	**FN+ vs SN+**	**FN+ vs SN-**
EC:1.1.99.1	310	-1.1	0.1	-2.3
EC:2.6.1.5	162	0	0.5	0.1
EC:2.6.1.57	77	-0.1	0.9	0.6
EC:2.4.1.1	38	0.6	0.8	1.4
EC:3.2.1.1	279	0.3	0.4	0.3
EC:3.1.1.3	1734	-0.3	-0.9	-0.6

Table 2.A3 FPKM values and fold change for transcripts encoded for ATPases

contigID	FN+	FN-	SN+	SN-	EC	FPKM FN+	log2 FN+/FN-	log2 FN+/SN+	log2 FN+/SN-
contig-100_7308:541-1398	2	5	538	378	**EC:3.6.1.3**	2	1.6	8.3	7.8
contig-100_5520:17-607	2	7	715	612	**EC:3.6.1.3**	2	1.5	8.3	8.0
contig-100_2762:95-1692	5	9	71	180	**EC:3.6.1.3**	5	1.0	3.9	5.2
contig-100_5924:1287-1748	34	43	240	247	**EC:3.6.1.3**	34	0.4	2.8	2.9
contig-100_186:4802-6362	17	25	119	156	**EC:3.6.1.3**	17	0.5	2.8	3.2
contig-100_9352:5-571	4	9	25	31	**EC:3.6.1.3**	4	1.1	2.6	2.9
contig-100_150:2822-4086	47	107	256	321	**EC:3.6.1.3**	47	1.2	2.4	2.8
contig-100_7483:65-1414	90	134	477	310	**EC:3.6.1.3**	90	0.6	2.4	1.8
contig-100_3981:656-1456	26	27	134	131	**EC:3.6.1.3**	26	0.1	2.4	2.4
contig-100_150:1714-2391	23	44	119	134	**EC:3.6.1.3**	23	0.9	2.4	2.5
contig-100_150:607-1266	39	68	197	195	**EC:3.6.1.3**	39	0.8	2.3	2.3
contig-100_9108:472-1068	57	58	244	150	**EC:3.6.1.3**	57	0.0	2.1	1.4
contig-100_11187:85-846	20	56	80	347	**EC:3.6.1.3**	20	1.5	2.0	4.1
contig-100_309:3647-5893	51	78	203	297	**EC:3.6.1.3**	51	0.6	2.0	2.5
contig-100_8783:531-1187	27	40	93	89	**EC:3.6.1.3**	27	0.6	1.8	1.7
contig-100_8508:179-1072	8	7	27	27	**EC:3.6.1.3**	8	-0.2	1.8	1.8
contig-100_9200:503-901	19	50	63	89	**EC:3.6.1.3**	19	1.4	1.8	2.3
contig-100_1730:7-3315	21	29	68	76	**EC:3.6.1.3**	21	0.5	1.7	1.9
contig-100_3665:419-2454	21	19	65	101	**EC:3.6.1.3**	21	-0.1	1.6	2.3
contig-100_10561:25-638	15	12	44	43	**EC:3.6.1.3**	15	-0.3	1.6	1.5
contig-100_1811:2418-2939	14	26	36	43	**EC:3.6.1.3**	14	0.9	1.4	1.7
contig-100_2472:1072-1512	34	50	89	138	**EC:3.6.1.3**	34	0.5	1.4	2.0
contig-100_213:290-1246	36	117	77	160	**EC:3.6.1.3**	36	1.7	1.1	2.2
contig-100_10425:32-811	38	10	70	1	**EC:3.6.1.3**	38	-2.0	0.9	-5.3
contig-100_7158:22-1418	39	6	52	2	**EC:3.6.1.3**	39	-2.7	0.4	-4.2
contig-100_948:79-2019	54	8	72	1	**EC:3.6.1.3**	54	-2.7	0.4	-5.3
contig-100_5308:213-1888	16	8	20	2	**EC:3.6.1.3**	16	-1.1	0.3	-3.3
contig-100_10292:20-850	493	152	578	538	**EC:3.6.1.3**	493	-1.7	0.2	0.1
contig-100_2690:756-1310	17	2	19	1	**EC:3.6.1.3**	17	-2.8	0.2	-5.0
contig-100_8852:713-1180	397	129	412	441	**EC:3.6.1.3**	397	-1.6	0.1	0.2
contig-100_542:142-5340	12	1	10	2	**EC:3.6.1.3**	12	-3.1	-0.2	-2.7
contig-100_2690:9-632	48	8	41	1	**EC:3.6.1.3**	48	-2.5	-0.2	-6.1
contig-100_2690:1547-2845	40	5	31	0	**EC:3.6.1.3**	40	-3.0	-0.4	-9.2
contig-100_514:3782-5335	24	9	18	6	**EC:3.6.1.3**	24	-1.3	-0.4	-2.1
contig-100_12078:270-785	1	1	0	4	**EC:3.6.1.3**	1	-0.3	-0.6	2.6
contig-100_17017:44-501	85	35	53	29	**EC:3.6.1.3**	85	-1.3	-0.7	-1.6
contig-100_550:296-1897	6	17	2	2	**EC:3.6.1.3**	6	1.6	-1.3	-1.4
contig-100_11812:63-807	137	114	47	76	**EC:3.6.1.3**	137	-0.3	-1.5	-0.9
contig-100_16157:100-563	234	138	78	133	**EC:3.6.1.3**	234	-0.8	-1.6	-0.8
contig-100_3864:255-2380	81	20	27	4	**EC:3.6.1.3**	81	-2.0	-1.6	-4.3
contig-100_11959:7-786	305	173	92	188	**EC:3.6.1.3**	305	-0.8	-1.7	-0.7
contig-100_15630:37-584	153	83	44	64	**EC:3.6.1.3**	153	-0.9	-1.8	-1.3
contig-100_13619:48-676	71	31	18	20	**EC:3.6.1.3**	71	-1.2	-2.0	-1.8
contig-100_13535:297-695	269	314	64	139	**EC:3.6.1.3**	269	0.2	-2.1	-1.0
contig-100_11874:38-829	60	18	13	12	**EC:3.6.1.3**	60	-1.7	-2.2	-2.3
contig-100_6:24-13086	67	19	11	13	**EC:3.6.1.3**	67	-1.8	-2.6	-2.3
contig-100_11049:29-418	57	19	8	12	**EC:3.6.1.3**	57	-1.6	-2.8	-2.2
contig-100_2386:2609-3091	57	79	6	28	**EC:3.6.1.3**	57	0.5	-3.3	-1.0
contig-100_662:212-5114	331	129	31	40	**EC:3.6.1.3**	331	-1.4	-3.4	-3.1
contig-100_589:152-643	1089	553	89	170	**EC:3.6.1.3**	1089	-1.0	-3.6	-2.7
contig-100_2693:97-2651	863	401	65	121	**EC:3.6.1.3**	863	-1.1	-3.7	-2.8
contig-100_3741:301-2334	202	47	14	22	**EC:3.6.1.3**	202	-2.1	-3.8	-3.2
contig-100_112:5203-7668	212	450	560	863	**EC:3.6.3.9**	212	1.1	1.4	2.0
contig-100_10686:3-683	66	160	212	310	**EC:3.6.3.6**	66	1.3	1.7	2.2
contig-100_8061:1-483	114	268	339	493	**EC:3.6.3.6**	114	1.2	1.6	2.1
contig-100_3090:1255-2283	0	1	7	1	**EC:3.6.3.2**	0	9.7	12.8	10.1

Chapter 3

Superior triacylglycerol (TAG) accumulation in starchless mutants of *Scenedesmus obliquus* (I): mutant generation and characterization

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Microalgae are a promising platform for producing neutral lipids, to be used in the application for biofuels or commodities in the feed and food industry. A very promising candidate is the oleaginous green microalga *Scenedesmus obliquus*, because it accumulates up to 45% w/w triacylglycerol (TAG) under nitrogen starvation. Under these conditions, starch is accumulated as well. Starch can amount up to 38% w/w under nitrogen starvation, which is a substantial part of the total carbon captured. When aiming for optimized TAG production, blocking the formation of starch could potentially increase carbon allocation towards TAG. In an attempt to increase TAG content, productivity and yield, starchless mutants of this high potential strain were generated using UV mutagenesis. Previous studies in *Chlamydomonas reinhardtii* have shown that blocking the starch synthesis yields higher TAG contents, although these TAG contents do not surpass those of oleaginous microalgae yet. So far no starchless mutants in oleaginous green microalgae have been isolated that result in higher TAG productivities. Five starchless mutants have been isolated successfully from over 3,500 mutants. The effect of the mutation on biomass and total fatty acid (TFA) and TAG productivity under nitrogen-replete and nitrogen-depleted conditions was studied. All five starchless mutants showed a decreased or completely absent starch content. In parallel, an increased TAG accumulation rate was observed for the starchless mutants and no substantial decrease in biomass productivity was perceived. The most promising mutant showed an increase in TFA productivity of 41% at 4 days after nitrogen depletion, reached a TAG content of 49.4% (% of dry weight) and had no substantial change in biomass productivity compared to the wild type. The improved *S. obliquus* TAG production strains are the first starchless mutants in an oleaginous green microalga that show enhanced TAG content under photoautotrophic conditions. These results can pave the way towards a more feasible microalgae-driven TAG production platform.

3.1 Introduction

With decreasing fossil fuel deposits and an increasing world population, the need for alternative renewable food and energy resources such as biofuels is stronger than ever. Several crops are considered for both edible oils and biofuels production such as rapeseed, jatropha, corn, soybean and palm oil (Chisti, 2007; Niyogi, 2000). However, for food and energy purposes, these crops need to be produced on a massive scale (Wijffels et al., 2010). This will dramatically impact the available fertile agricultural land for food production (Chisti, 2007). Alternatively, microalgae are photoautotrophic microorganisms acknowledged for the production of bulk chemicals, biofuels, food, feed and nutraceuticals (Draaisma et al., 2013; Wijffels et al., 2010), and can be cultivated on saline and non-fertile grounds.

Several studies have been carried out on the production of triacylglycerol (TAG) from microalgae for the generation of biodiesel or edible oils (Chisti, 2007; Draaisma et al., 2013; Haik et al., 2011). Microalgae show a high potential as TAG producing organisms compared to other photosynthetic organisms (Chisti, 2007; Hu et al., 2008). The mechanism of TAG production in microalgae is not yet fully understood, but the generally accepted hypothesis is that when microalgae are exposed to unfavorable growth conditions, such as extreme pH, nutrient limitation or high salinity, they channel the excess energy from light into storage compounds such as starch and TAG (Breuer et al., 2013b; Hu et al., 2008; Santos et al., 2012). These storage compounds serve as electron sinks, that alleviate an over-reduced photosystem, and in this way prevent the formation of reactive oxygen species, which can induce photooxidative stress (Hu et al., 2008; Ledford and Niyogi, 2005).

The rate at which starch and TAG are accumulated *in vivo* under growth-limiting conditions is dependent on the microalgae species and cultivation conditions used (Breuer et al., 2012). *Chlamydomonas reinhardtii* is known for the production of large amounts of starch granules up to 45% of cell dry weight (DW) (Li et al., 2010a), whereas oleaginous microalgae such as *Scenedesmus obliquus* (recently suggested to be reclassified to *Acutodesmus obliquus* (Krienitz and Bock, 2012)) accumulate, besides starch, lipid bodies rich in TAG (Breuer et al., 2012; Vigeolas et al., 2012). Generally, starch is accumulated from the onset of starvation and TAG accumulation is commenced a few hours to days later (Siaut et al., 2011). Although *S. obliquus* is referred to as an oleaginous species, other carbon containing compounds are produced as well. In the study of Breuer *et al*., the predicted carbohydrate content of *S. obliquus* is 40 to 60% of the dry weight (Breuer et al., 2012) and TAG contents of 30 to 45% of the dry weight were observed (Breuer et al., 2013b). Furthermore, the total fatty acid (TFA) composition is very suitable for the production of biodiesel due to the low content of linolenic and other polyunsaturated

fatty acids, and the relative abundance of saturated and monounsaturated fatty acids (Gouveia and Oliveira, 2009).

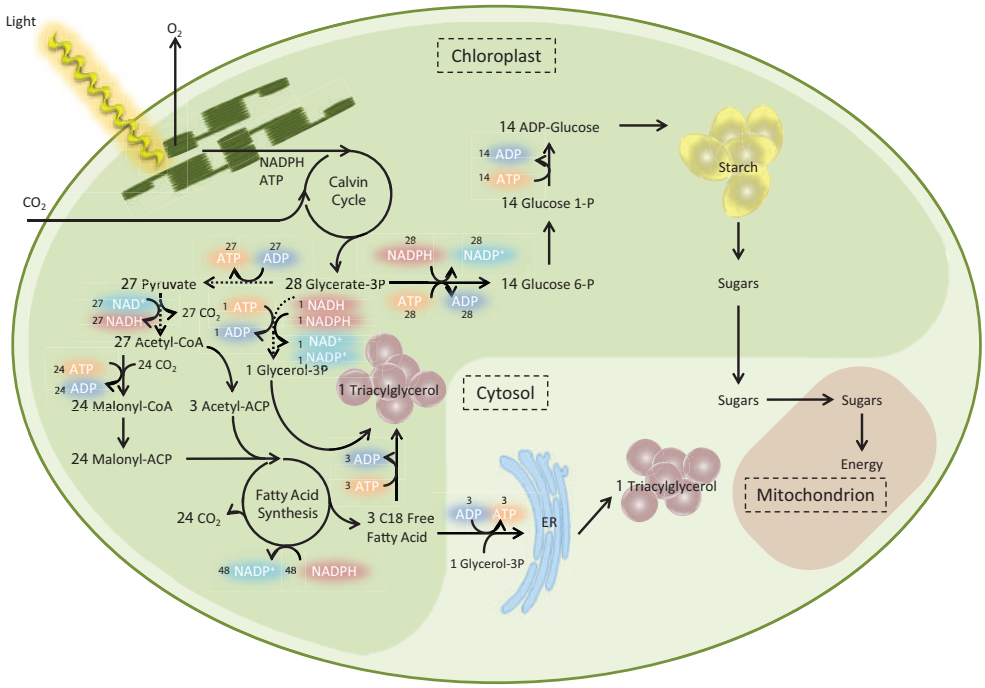

Figure 3.1 Simplified triacylglycerol and starch metabolism in green microalgae. Not all reactions are shown, and the cofactors upstream of 3PG are not considered. The dashed lines are reactions that take place in the cytosol. From 28 molecules of 3PG, one molecule of C18 TAG can be formed or 14 molecules of glucose in a starch matrix. Two possible ways for the formation of TAG molecules are shown following the postulated route in the chloroplasts or over the ER membranes in the cytosol (Johnson and Alric, 2013; Radakovits et al., 2010; Scott et al., 2010). 3PG, 3-phosphoglycerate; ER, endoplasmic reticulum; TAG, triacylglycerol.

Although there is no complete understanding of the mechanism behind carbon partitioning and the switch from starch towards TAG production, parts of the puzzle are known. Figure 3.1 shows a schematic overview of the carbon partitioning in microalgae. There is a common denominator in the carbon metabolism, the C3 pool, such as 3-phosphoglycerate (3PG) and glyceraldehyde 3-phosphate (GAP). The competition for those substrates is commonly referred to as carbon partitioning. Starch functions as a primary energy investment that can be stored during the day and used at night to provide energy for key metabolic processes (de Winter et al., 2013). TAG is considered as a secondary energy and electron sink (Hu et al., 2008), although not much is known about the regeneration of the energy stored in TAG molecules after a period of growth inhibition.

Since both TAG and starch compete for carbon, through the common C3 precursors, it was hypothesized that when the pathway towards starch formation is blocked, the carbon and energy flux towards TAG molecules is enhanced. This hypothesis was confirmed in starchless mutants (Slm) of *C. reinhardtii* (Li et al., 2010b; Wang et al., 2009). Li *et al.* compared TFA accumulation in wild type (wt) *C. reinhardtii* with five low starch or starchless mutants under nitrogen-limited conditions (I7, JV45J, BAFJ5, CR102, BAFJ6 (Ball et al., 1991; Colleoni et al., 1999; Mouille et al., 1996; Wattebled et al., 2003; Zabawinski et al., 2001)). They showed that the starch content of these mutants was lowered or even completely absent resulting in an increased TFA content compared to the wild type (Li et al., 2010a). The starchless mutants showed that the TAG content could be increased from 0.5 to 20.5% (% of DW). Although the TAG content increased, growth kinetics of the starchless *C. reinhardtii* strains are severely subdued by the inserted mutation (Li et al., 2010b), resulting in decreased TAG productivity (Li et al., 2010a).

Besides the generation of starchless mutants using random mutagenesis, more directed approaches have been used as well. However, there are currently not many examples of successful enhancement of TAG content in microalgae using directed genetic engineering. Attempts to overcome bottlenecks in the TAG synthesis pathway, including overexpression of the enzymes acetyl-CoA carboxylase (ACCase) and the acyl-transferase step, 1,2-diacylgycerol acyltransferase (DGAT), did not yield enhanced TAG contents (Dunahay et al., 1996; La Russa et al., 2012). Recently Trentacoste *et al.* (Trentacoste et al., 2013) showed that the knockdown of genes involved in the lipid catabolism can increase the TAG content in the diatom *Thalassiosira pseudonana*.

The fact that *C. reinhardtii* is not an oleaginous microalga by nature, but a starch producing microalga under unfavorable growth conditions, probably does not make this species the most suitable candidate to study lipid accumulation in starchless mutants. In this study, starchless mutants from the oleaginous microalga *S. obliquus* were generated to study the effect of blocked starch synthesis on TAG accumulation in a microalgae that by nature have the ability to accumulate high contents of TAG. Increasing the TAG productivity is an important step to enable a feasible microalgae-driven TAG production platform.

3.2 Materials and Methods

3.2.1 Strain and mutant generation

The *S. obliquus* wild type strain (UTEX 393) (recently suggested to be reclassified to *A. obliquus* (Krienitz and Bock, 2012)) was acquired from the Culture Collection of Algae at the University of Texas at Austin (UTEX; Austin, TX, USA). The culture was maintained on Tris-Acetate-Phosphate (TAP) medium as described by Gorman and Levine (Gorman

and Levine, 1965), with addition of 15 g/L agar for plates. Approximately 20,000 cells in late log phase (1 x 10^7 cells/mL) were plated on TAP agar plates and air-dried for 30 min before UV treatment. Subsequently, the cultures were exposed to 40,000 µJ cm^{-1} of monochromatic UV light with a wavelength of 254 nm (CL-1000 UV crosslinker; UVP, Upland, CA, USA) at room temperature, resulting in a 5 to 10% survival rate. The total dose of radiation was determined by the UV sensor equipped with the instrument. After irradiation, plates were stored overnight in the dark to reduce light-induced repair mechanisms. Subsequently, plates were incubated in 50 µmol m^{-2} s^{-1} light under continuous illumination at 25°C for 10 days, after which single colonies were picked and transferred to fresh TAP plates and categorized in a grid. As a positive control, every plate contained a patch of the *C. reinhardtii* starch mutant BAFJ5 (Zabawinski et al., 2001). Plates were cultivated under the same conditions for 5 days, after which all colonies were replica plated to TAP-N (NH_4Cl was omitted and substituted by an equimolar amount of KCl) plates in the same grid. After 9 days of incubation the colonies were screened for the presence of starch by applying an iodine vapor staining (Work et al., 2010). Colonies that did not stain purple after the iodine vapor treatment were considered as potential starchless mutants, and selected for further analysis.

3.2.2 Triacylglycerol induction in starchless mutants of S. obliquus

Pre-cultures were maintained photoautotrophically under day-night regime (16 h:8 h) at 25°C, 125 rpm and under an incident light intensity of 40 µmol m^{-2} s^{-1}. The wild type and all starchless mutants were able to cope with multiple serial dilutions. The photoautotrophic culture medium that was used during the experiments comprised of: 33.6 mM KNO_3 (in nitrogen-replete medium, N+) or 33.6 mM KCl (in nitrogen-depleted medium, N-); 0.7 mM Na_2SO_4; 100 mM 2-(4-(2-hydroxyethyl)piperazin-1-yl)ethanesulfonic acid (HEPES); 1 mM $MgSO_4 \cdot 7H_2O$; 0.5 mM $CaCl_2 \cdot 2H_2O$; 2.5 mM K_2HPO_4; 10 mM $NaHCO_3$; 28 µM NaFeEDTA; 80 µM $Na_2EDTA \cdot 2H_2O$; 19 µM $MnCl_2 \cdot 4H_2O$; 4 µM $ZnSO_4 \cdot 7H_2O$; 1.2 µM $CoCl_2 \cdot 6H_2O$; 1.3 µM $CuSO_4 \cdot 5H_2O$; and 0.1 µM $Na_2MoO_4 \cdot 2H_2O$. The pH was adjusted to pH 7.0 with NaOH. To study the growth of the wild type and all Slm, two cultivation experiments were conducted in which all strains (triplicates) were exposed to nitrogen-replete photoautotrophic conditions under 16 h:8 h day-night regime for 5 to 7 days in a 6-well plate containing 5 mL culture volume. Growth was followed by OD_{750} measurements using the Tecan Safire plate reader (Tecan Austria GmbH, Grödig, Austria). To evaluate the TAG producing capacity of the selected mutants, cultures were maintained in 115 mL photoautotrophic medium in 250 mL glass Erlenmeyer flasks under an incident light intensity of 60 µmol m^{-2} s^{-1} (continuous illumination, Grolux fluorescent tubes, Sylvania F36W/GRO) at 25°C in an Multitron incubator shaker (Infors AG, Bottmingen, Switzerland). Agitation was kept at 125 rpm and the headspace was enriched to 5% CO_2. Two flasks of wild type *S. obliquus*, as well as all selected mutants, were grown under nitrogen-replete conditions to a biomass concentration of 1.5 g/L. Duplicate flasks were pooled to provide sufficient starter

material, and divided into four aliquots with an equal cell concentration. The aliquots were washed (centrifuged for 5 minutes at 400 × *g*) two times with either medium containing 33.6 mM KNO_3 (N+) or medium with an equimolar amount of KCl (N-), and subsequently resuspended in either nitrogen-replete or nitrogen-depleted medium. This step is considered as T0. As such, duplicate cultures were available for all tested mutants and the wild type, under both N- and N + conditions. Culture development was studied over a period of 12 days. Samples were taken at 0, 48, 70, 92, 138 and 285 hours, to evaluate optical density, dry weight and biomass composition. For every time point, TFA and starch contents were analyzed, and for the last time point the TAG content was determined. For all parameters biological replicates were examined.

3.2.3 Determination of dry weight concentration

Dry weight concentrations were determined at every time point on biological replicates. Around 1.5 mg of biomass was filtered through pre-dried (100°C overnight) and preweight Whatman glass fiber filter paper (GF/F; Whatman International Ltd, Maidstone, UK). The filter was washed with 50 mL of filtered demineralized water and subsequently dried overnight at 100°C before weighing.

3.2.4 Total fatty acid analysis

TFA extraction and quantification were executed as described by Breuer *et al*. (Breuer et al., 2013a) with the following adjustments. Around 5 mg of pellet was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) and lyophilized overnight. Freeze-dried cells were disrupted by a 30 min bead beating step in the presence of a chloroform:methanol mixture (1:1.25) to extract the lipids from the biomass. Next, 48.6 µg/mL tripentadecanoin (T4257; Sigma-Aldrich, St Louis, MO, USA) internal standard was added to the extraction mixture to enable fatty acid quantification. Methylation of the fatty acids to fatty acid methyl esters (FAMEs) and the quantification of the FAMEs were performed as described by Breuer *et al.* (Breuer et al., 2013a). TFA concentration was calculated as the sum of all individual fatty acids.

3.2.5 Triacylglycerol analysis

The method used for the quantification of TAG was similar to the TFA analysis method with the following modifications. After the TFA extraction, the chloroform methanol mixture was evaporated under N_2 gas and the TFA fraction was dissolved in 1 mL hexane and separated based on the polarity using a Sep-Pak Vac silica cartridge (6 cc, 1,000 mg; Waters, Milford, MA, USA) prewashed with 6 mL of hexane. The neutral TAG fraction was eluted with 10 mL of hexane-diethyl ether (87:13% v/v). The polar lipid fraction containing the glycolipids and phospholipids remained in the silica cartridge. Subsequently, the TAG fraction was methylated and analyzed as described in the TFA analysis section.

3.2.6 Starch determination

The starch content of *S. obliquus* was analyzed by enzymatic degradation of starch to glucose using the thermostable α-amylase and amyloglucosidase enzymes from the Total Starch assay (Megazyme International, Wicklow, Ireland) using the protocol as described by de Winter *et al.* (de Winter et al., 2013). The following modifications to the protocol were made. Around 10 mg of biomass was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals) and lyophilized overnight. Freeze-dried cells were disrupted by bead beating in the presence of 80% ethanol. Starch was converted to glucose using α-amylase and amyloglucosidase enzymes. Subsequently glucose was colored and absorbance was measured against a D-glucose calibration control series at a wavelength of 510 nm.

3.3 Results and discussion

Previous studies on green microalgae starchless mutants have focused mainly on the model species *C. reinhardtii*. The most promising starchless mutant known in *C. reinhardtii* is the BAFJ5 mutant containing a recessive mutation on the *sta6-1* gene encoding the small subunit of ADP-glucose pyrophosphorylase (AGPase) (Zabawinski et al., 2001). Under high light and nitrogen-limiting growth conditions, BAFJ5 accumulates up to 20.5% TAG (% of DW) under mixotrophic conditions (acetate) and around 14% under phototrophic conditions (Li et al., 2010a). *C. reinhardtii* is not an oleaginous microalga by nature but blocking the starch metabolism induced the accumulation of TAG molecules under nitrogen-depleted conditions (Li et al., 2010a; Li et al., 2010b). In this study we generated starchless mutants from the oleaginous green microalga *S. obliquus* and studied the effect on the TAG content of the mutants.

3.3.1 S. obliquus mutant screening

Over 3,500 potential mutants of *S. obliquus* were obtained after UV irradiation, which were all screened for the absence of starch using the iodine vapor method. Wild type cells containing starch stained dark purple when exposed to iodine vapor (Figure 3.2A). Starchless mutants that stained pale green instead of purple were considered as potential starchless mutants and selected for further study. *C. reinhardtii* BAFJ5 was used as a positive control to check for reduced starch levels. In total, six colonies showed a reduced or absent coloring and were selected for further study (Figure 3.2A); Slm5 was not included in this study because the growth under replete conditions was retarded in such a way that it could not be compared to the other strains. The five potential starchless *S. obliquus* mutants, as well as the wild type, were exposed to nitrogen starvation in a 100 mL shake flask cultivation experiment, to evaluate growth kinetics and biomass compositions. The biomass was examined on TFA, neutral lipid content (TAG) and starch content. The composition of the TFA and TAG was determined based on the FAMEs.

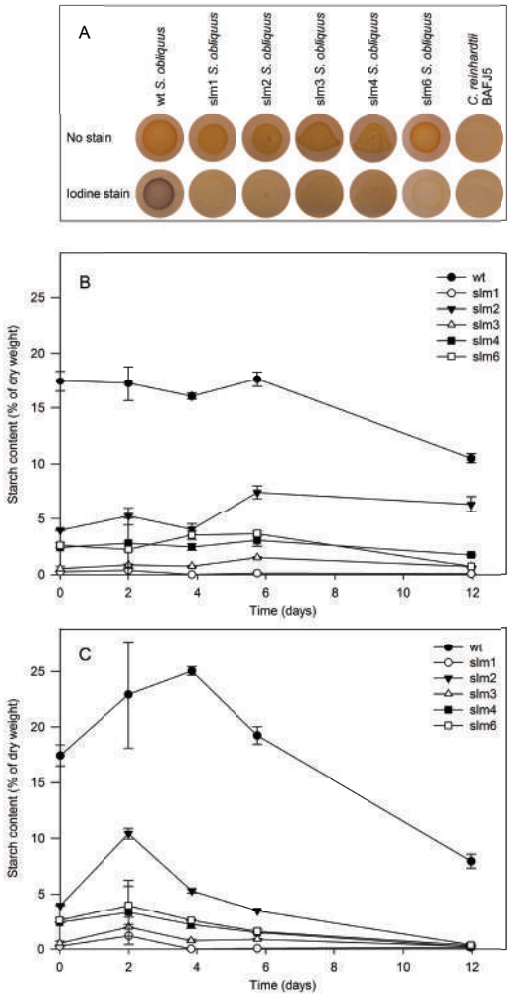

Figure 3.2 Starch screening and content determination. (A) Iodine vapor staining on starchless mutants. *C. reinhardtii* strain BAFJ5 was included as a control. The staining was performed after 9 days of nitrogen deprivation (TAP-N plates) to enhance the visibility of the iodine staining which is normally interfered by the chlorophyll. Starch content (% of DW) for (B) nitrogen-replete and (C) nitrogen-depleted conditions over time. The values are derived from biological duplicate cultures. Deviation from the duplicate average is indicated by the error bars. DW, dry weight; slm, starchless mutant; TAP, Tris-Acetate-Phosphate; wt, wild type.

3.3.2 S. obliquus mutant biomass accumulation

Under nitrogen-replete conditions wild type *S. obliquus* accumulated biomass in a linear manner and reached around five times the start concentration after 12 days. Under nitrogen-depleted conditions the biomass increased around three times (Figure 3.3). This was also observed by Breuer *et al*. (Breuer et al., 2012). Interestingly, similar growth kinetics were observed in the five selected mutants, and all strains approached the final biomass concentration of the wild type (4.28 ± 0.04 g/L under nitrogen-replete conditions). Minor differences were observed in biomass concentration under nitrogen-replete conditions, and all mutants showed a slightly lower biomass concentration compared to the wild type. Under nitrogen-depleted conditions all *S. obliquus* potential starchless mutants had the same final biomass concentrations as the wild type (2.43 ± 0.04 g/L) except Slm1 (2.72 ± 0.03 g/L), which was able to accumulate more biomass than the wild type.

Another way to study the growth kinetics is to examine the biomass productivity. In Table 3.1 the average and maximal volumetric biomass productivities (mg L^{-1} day^{-1}) are shown. Under nitrogen-replete growth, Slm2 had a higher average productivity compared to the wild type, with 322 ± 5 mg L^{-1} day^{-1} and 295 ± 2 mg L^{-1} day^{-1}, respectively. Under nitrogen-depleted conditions, Slm2, Slm3 and Slm4 exhibited a similar biomass productivity compared to the wild type (140 ± 5 mg L^{-1} day^{-1}) ranging from 137 to 147 mg L^{-1} day^{-1}. Slm6 had the lowest average biomass productivity, while Slm1 showed the highest average biomass productivity, with 129 ± 1 mg L^{-1} day^{-1} and 166 ± 2 mg L^{-1} day^{-1}, respectively (Table 3.1).

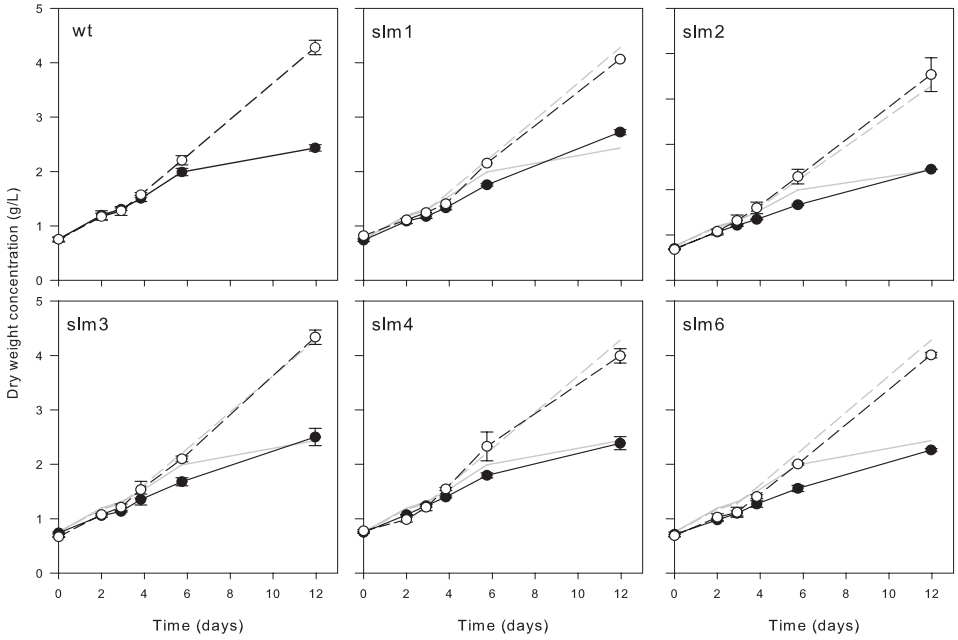

Figure 3.3 Growth curves of S. obliquus wild type and five selected starch mutants. Solid lines and filled circles represent strains cultivated under nitrogen-depleted conditions; dotted lines and open circles represent nutrient-replete conditions. The gray-scale lines visualize the wild type growth curve in the starchless mutant plots. The values are derived from duplicate cultures. Deviation from the duplicate average is indicated by the error bars.

Starch is an energetically low-cost storage metabolite, which through respiration functions as an energy source when photosynthesis is impaired. As starch is such an important storage component, its absence in starch mutants is expected to impair growth to a certain extent, as was observed in *C. reinhardtii* (Li et al., 2010a). Li *et al.* showed a 30% growth reduction compared to the wild type under photoautotrophic conditions for the starchless mutant BAFJ5. The *S. obliquus* starchless mutants showed minor differences

in the growth kinetics between the mutants and the wild type. This indicates that the metabolism of the mutants is not severely imbalanced and that *S. obliquus* found other ways to supply energy when photosynthesis is impaired, most likely through the use of the stored TAG.

Table 3.1 Volumetric productivities of *S. obliquus* wild type and five selected starchless mutants

		Volumetric biomass productivity (mg L^{-1} day^{-1})		Volumetric TFA productivity (mg L^{-1} day^{-1})		Volumetric TAG productivity (mg L^{-1} day^{-1})
		Average	Maximum	Average	Maximum	Average
Wild type	N+	295 ± 2	334 ± 25	30 ± 2	42 ± 2	4 ± 0
	N-	140 ± 5	**255^{b} ± 4**	104 ± 5	138 ± 5	95 ± 3
Slm 1	N+	271 ± 1	390 ± 24	35 ± 1	44 ± 2	3 ± 0
	N-	**166^{b} ± 2**	222 ± 20	**125^{b} ± 2**	**173^{b} ± 9**	**112^{b} ± 3**
Slm 2	N+	**322^{a} ± 5**	362 ± 24	**42^{a} ± 5**	54 ± 7	12^{a} ± 1
	N-	146 ± 0	182 ± 22	101 ± 0	145 ± 2	94 ± 1
Slm 3	N+	307 ± 1	360 ± 9	42 ± 1	**57^{a} ± 1**	3 ± 0
	N-	147 ± 1	240 ± 69	107 ± 1	170 ± 14	98 ± 2
Slm 4	N+	269 ± 2	**408^{a} ± 87**	35 ± 2	46 ± 4	4 ± 0
	N-	137 ± 1	208 ± 24	105 ± 1	140 ± 6	95 ± 0
Slm 6	N+	278 ± 2	322 ± 8	35 ± 2	46 ± 4	7 ± 0
	N-	129 ± 1	187 ± 8	82 ± 1	115 ± 6	76 ± 1

Average productivities are calculated as the amount of TFA formed (mg) over the course of the experiment (12 days) per liter culture medium. Maximal productivities represent the highest productivity values between two consecutive time points. Average productivities are calculated for biomass, TFA and TAG. Maximal productivities are calculated for biomass and TFAs. Bold numbers represent the highest values for each parameter. [a]Highest value for nitrogen-replete cultivated strains; [b]highest value for nitrogen-depleted cultivated cultures. Average values of biological duplicate cultures are shown followed by the distance to the average.

3.3.3 Biomass composition analysis

The oleaginous microalga *S. obliquus* is known for its ability to produce high amounts of TAG under suboptimal growth conditions (Breuer et al., 2012; Vigeolas et al., 2012). Upon nitrogen starvation *S. obliquus* cells start to accumulate neutral lipid droplets inside the cell containing TAG molecules. Besides the accumulation of TAG molecules, starch is also accumulated (Vigeolas et al., 2012). It is hypothesized that mutants unable to accumulate starch will show an increased TAG content compared to the wild type, to counter the extra excess energy that cannot be stored in starch anymore upon growth inhibition. The major metabolite of interest for lipid production in microalgae is the TFA content. Even

more so the TAG content that represents the neutral lipid fraction is of interest for the applications in food, feed and fuels.

3.3.4 Starch

The wild type *S. obliquus* cells contained on average 17% of their dry weight as starch under nitrogen-replete growth in the first 6 days (Figure 3.2B). When cells were exposed to nitrogen-depleted conditions, the starch level increased to 25% of the dry weight within the first 4 days after medium replacement. Starch accumulation seemed to be a transient storage mechanism, since the starch content first increased and subsequently decreased to 10% after 12 days of nitrogen-depleted growth. All mutants showed a similar starch accumulation profile, but had a much lower starch content with respect to the wild type. Slm1 and Slm3 showed the lowest starch content with a maximum of 1.2% and 2.0% of the dry weight, respectively (Figure 3.2C). These values are within the detection limit of the starch determination assay. This indicates that in these mutants the starch synthesis pathway was successfully impaired.

Several oleaginous microalgae are known to accumulate carbohydrates during the first days of nitrogen-depleted conditions (Draaisma et al., 2013; Johnson and Alric, 2013; Vigeolas et al., 2012). The major form of carbohydrates that is accumulated is starch, and it is accepted that this metabolite functions as a primary carbon and electron storage compound. Starch serves as a primary storage compound, since the electrons required per unit of biomass are lower compared to TAG and proteins, which are more reduced than carbohydrates.

3.3.5 Total fatty acid content and composition

In Figure 3.4 it can be observed that under nitrogen-depleted conditions the TFA content is increased, and under nitrogen-replete growth no increase in the TFA content is observed. Under nitrogen-replete conditions all starchless mutants showed a higher TFA content compared to the wild type. An explanation could be that the absence of starch in the biomass makes the contribution of TFA compared to the dry weight slightly higher, therefore the relative TFA content would be higher. Another option could be that the internal TFA and TAG content was increased due to the blocked starch synthesis. As a result of this impairment, the TFA content of all mutants increased after nitrogen starvation. All mutants showed an increased TFA accumulation rate during the first 6 days of nitrogen-depleted conditions. After 6 days the differences between the wild type and the mutants decreased, resulting in similar TFA contents after 12 days. Slm1 showed an increased TFA content (57.3 ± 0.4%) compared to the wild type (53.9 ± 1.8%) and Slm6 seemed to lag behind (47.1 ± 0.3%). The TFA content of the starchless mutants showed a higher TFA accumulation rate after nitrogen starvation was induced, shortening the cultivation time in a batch culture.

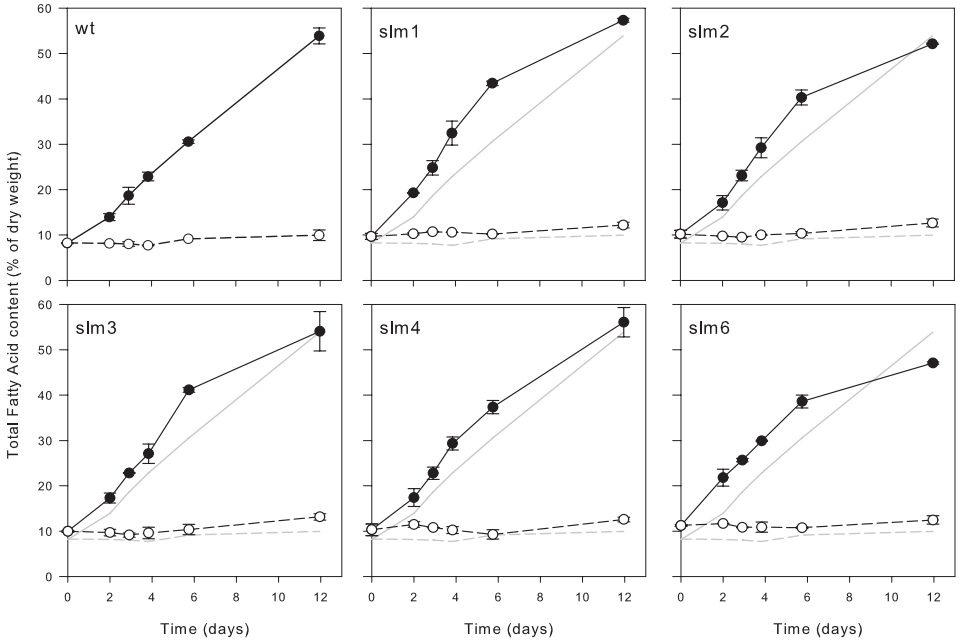

Figure 3.4 Total fatty acid content of *S. obliquus* wild type and five selected starch mutants. Values presented as percentage of dry weight. The dotted line and the solid line represent nitrogen-replete and nitrogen-depleted conditions, respectively. The gray-scale lines in the plots represent the wild type TFA content. The values are derived from duplicate cultures. Deviation from the duplicate average is indicated by the error bars.

The TFA content only partly shows the capacity of the selected starchless mutants to produce lipids. In order to assess the potential for lipid production of each strain, TFA productivity should be compared, since the TFA productivity represents the capacity of the strain to convert received light, which was the same for each culture, into the desired compound per unit of time. The differences in biomass concentration are taken into account, enabling a fair comparison of the TFA production by all strains tested. The initial biomass concentration at the start of nitrogen depletion was the same for all strains, thus an equal amount of light per gram of dry weight was supplied. The average productivity at which fatty acids were synthesized was higher under nitrogen-depleted conditions than under nitrogen-replete conditions (Table 3.1), with Slm1 showing the highest average TFA productivity with respect to the wild type, 125 ± 2 mg L^{-1} day^{-1} compared to 104 ± 5 mg L^{-1} day^{-1}, respectively; this corresponds to 20% more TFA than the wild type. Figure 3.5C shows that Slm1 has a higher maximal TFA productivity compared to the wild type at every time point. The largest difference in maximal volumetric productivity is found between day 3 and day 4 after nitrogen starvation started, and Slm1 accumulated 41% more TFA compared to the wild type (154 ± 40 mg L^{-1} day^{-1} and 109 ± 14 mg L^{-1} day^{-1},

respectively) (Figure 3.5B,C). The second largest difference is at day 2, when Slm1 accumulates 32.7% more TFA than the wild type (69 ± 4 mg L^{-1} day^{-1} and 52 ± 1 mg L^{-1} day^{-1}, respectively). The maximum productivity, representing the productivity between two consecutive time points, was 25% higher in Slm1 (173 ± 9 mg L^{-1} day^{-1}) compared to the wild type (138 ± 5 mg L^{-1} day^{-1}).

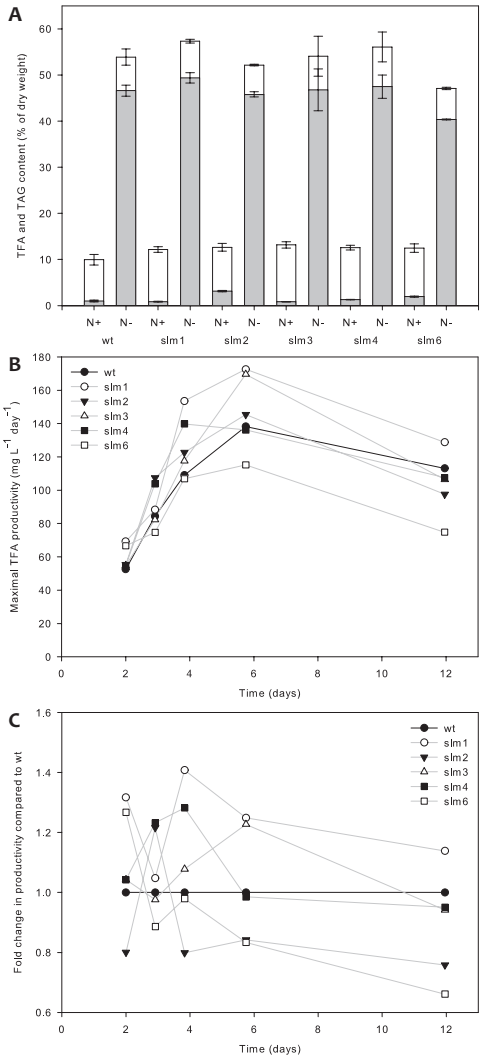

Figure 3.5 Triacylglycerol content and total fatty acid productivity. (A) Lipid content 12 days after nitrogen depletion. TAG (gray bars) and polar lipid (glycolipids and phospholipids) (white bars) content as percentage of dry weight. The values are derived from duplicate cultures. Deviation from the duplicate average is indicated by the error bars. (B) Productivity calculated between two consecutive time points in mg L^{-1} day^{-1}. Wild type is represented by solid black line and the mutants are represented by gray lines. (C) Fold change in productivity (calculated between two consecutive time points) for all starchless mutants (gray-scale) compared to the wild type (solid line).

The TFA profiles of all strains were studied. The TFA and TAG fatty acid profiles are shown in Appendix Table 3.A1. There are no significant changes in the fatty acid profiles between the starchless mutants and wild type *S. obliquus*. This indicates that the fatty acid metabolism was not changed, and that the extra TFA and TAG formed in the mutants have the same composition and profile as wild type *S. obliquus*.

3.3.6 Triacylglycerol

The TAG content was determined at the final time point, day 12 (Figure 3.5A). This was done because only here sufficient biomass was available for the analysis. Assuming that there is no polar lipid degradation or formation during nitrogen depletion, the TAG content in the intermediate time points can be calculated by subtracting the polar lipid fraction observed at the end of the nitrogen-replete cultivation, from the measured TFA fraction at each time point. Figure 3.5A shows that the difference in polar lipid fraction between nitrogen-replete and nitrogen-depleted cultures is small. Under nitrogen-replete growth the TAG content was low and ranged from 0.9 ± 0.0% to 3.2 ± 0.1% of total dry weight. The wild type contained around 1 ± 0.1% TAG (% of DW), which supports previous observations (Breuer et al., 2012). Slm6 and Slm2 show a higher TAG content under nitrogen-replete growth conditions, with 2.0 ± 0.1% and 3.2 ± 0.1% (% of DW), respectively. This is in concurrence with our observation that throughout the entire nitrogen-replete experiment, starch mutants showed an elevated TFA content (Figure 3.4). *S. obliquus* wild type cells could accumulate 46.6 ± 0.9% of the cell dry weight in TAG, which represents 86% of the TFA content. Slm1 displayed a TAG content of 49.4 ± 0.8%, which represents 86% of the TFA content. Several studies have reported an inverse proportional relation between the lipid content and growth rate of the selected microalgae species. In other words, the higher the growth rate the lower the lipid content (% of DW), and vice versa (Griffiths and Harrison, 2009; Hu et al., 2008; Huo et al., 2012).

In this study we show that the oleaginous microalga *S. obliquus* is able to accumulate more neutral lipids when the starch synthesis is blocked without considerably changing the biomass productivity. These observations suggest that *S. obliquus* produces TAG directly from light and CO_2 and not from starch, and that the efficiency of light conversion to energy is not compromised. This is in contrast to other oleaginous microalgae. In the study of Li *et al. Pseudochlorococcum* sp. transient starchless mutants were studied (Li et al., 2011). The reduced starch content resulted in the reduction of TAG content, suggesting that in this species, starch functions as a feedstock for the subsequent production of TAG molecules. The same mechanism has been observed in higher plants (Periappuram et al., 2000). The results from this study show that when starch synthesis is blocked the TAG content and productivity increases. It is important to realize that the TAG accumulation mechanism is not the same in all oleaginous microalgae. The differences in phenotype and metabolism between species show the importance of carefully selecting a species with the right traits specific for the product of interest.

3.3.7 Industrial applications

In microalgae, starch can function as a primary carbon storage compound that is accumulated during the day and consumed during the night (de Winter et al., 2013; Ral et al., 2006). A similar phenomena is observed in the higher plant *Arabidopsis thaliana* in

which starch respiration is under circadian control to ensure a sufficient carbohydrate supply until the next day (Graf et al., 2010). Starchless mutants of *A. thaliana* are found to have a similar behavior compared to the wild type when exposed to very long days or continuous light, but have an impaired growth when exposed to normal diurnal light cycles (Caspar et al., 1985; Gibon et al., 2004). The starchless mutants of *S. obliquus* might therefore face problems to survive light-dark cycles. We therefore evaluated the growth of starchless mutants of *S. obliquus* under day-night cycles and found that this is not the case and all starchless mutants were able to grow in day-night cycles under replete conditions, although some starchless mutants showed a slightly altered growth rate (Appendix Figure 3.A1). It is very likely that another storage compound, likely TAG, is used as an energy and carbon source during the night.

The mutants generated by UV mutagenesis in this study need to be screened regularly for the stability of the mutation over multiple generations, as they do not contain a selection marker or any other trait to apply an external selection pressure to maintain the mutation. This is in contrast to transformants that are obtained by targeted transformation approaches or mutants obtained by insertion mutagenesis. A major advantage of UV-generated mutants is that for the large-scale outdoor cultivation and final use in food products no additional requirements and regulations are anticipated. The mutants presented in this work have shown to be stable over the course of 20 months. In particular, Slm1, with its high TAG production rate and non-impaired growth kinetics, compared to the wild type under both continuous as well as light-dark illumination, would be a suitable candidate for large-scale, outdoor TAG production.

3.4 Conclusion

Five starchless mutants of the oleaginous microalga *S. obliquus* were obtained by UV mutagenesis. Five starchless mutants were selected and showed a decreased or completely absent starch content. In parallel, an increased TAG accumulation rate was observed for the starchless mutants and no substantial decrease in biomass productivity was perceived. All mutants showed an increased TFA and neutral lipid productivity. Slm1 has a 41% higher TFA productivity compared to the wild type under phototrophic nitrogen-limiting conditions after 4 days of nitrogen depletion and reached a TAG content of 49.4% (% of DW). The increase in TFA is achieved without compromising the growth rates of the starchless mutants. Therefore, Slm1 should be considered as a potential production strain for TAG, since the increased lipid productivity shortens the batch runtime with respect to the wild type, bringing economical microalgae biofuel production one step closer.

Acknowledgements

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Appendix

Table 3.A1 Fatty acid composition expressed as % of total fatty acids (TFA) or fatty acids in TAG (TAG) 12 days after medium replacement. (A) Average fatty acid composition of TFA and TAG during nitrogen replete cultivation. (B) Fatty acid composition of TFA and TAG under nitrogen deplete conditions. Numbers given are averaged values (n=2) and the deviation from the duplicate average was less than 1% for all values except for the values that are indicated by an asterix, where the deviation was between 1% and 5%.

(A)	Wild type		Slm1		Slm2		Slm3		Slm4		Slm6	
	TFA	TAG	TFA	TAG	TFA	TAG	TFA	TAG	TFA	TAG	TFA	TAG
C16:0	16.2	11.2	16.0	11.2	16.2	12.5	16.3	13.1	16.6	15.4	14.9	10.6
C16:1	2.6	1.7	2.6	0.1	2.3	1.9	2.6	0.6*	2.5	2.0	2.3	1.8
C16:2	2.4	1.1	3.0	1.3	1.9	0.9	2.6	1.2	2.5	0.5	2.3	1.1
C16:3	3.4	1.4	4.4	1.7	5.5	1.5	3.6	1.2	4.1	1.4	3.6	0.7
C16:4	11.1	3.6	10.5	3.5	7.8	2.7	11.5	3.7	10.9	2.7	10.7	3.9
C18:0	0.6	2.2	0.5	2.2	0.7	1.3	0.6	3.0	0.8	2.2	0.6	1.5
C18:1	14.8	43.6	12.3	43.1	24.5	54.2	12.2	42.1	14.3	46.6*	17.1	47.0
C18:2	19.9	11.5	23.1	13.2	13.0	6.0	21.1	11.5	18.2	8.1	17.6	10.5
C18:3	18.7	10.2	18.7	11.1	10.3	11.6	18.9	11.6	19.8	11.1	19.6	11.1
C18:4	5.1	6.6	4.7	5.9	5.6	5.8	5.2	5.8	5.5	5.4	5.9	6.8
Other	5.2	6.8	4.2	7.5	12.2	1.5	5.4	6.2	5.0	4.5	5.6	5.0
(B)												
C16:0	16.5	16.2	16.4	16.2	18.0	18.0	17.8	17.7	17.9	17.8	15.8	15.4
C16:1	3.8	4.0	4.4	4.6	4.1	4.3	3.9	4.1	3.6	3.8	3.7	3.9
C16:2	2.8	2.6	2.9	2.7	2.4	2.3	2.6	2.5	2.0	2.0	2.8	2.7
C16:3	2.4	2.0	2.7	2.3	2.5	2.2	2.5	2.2	2.7	2.3	2.7	2.3
C16:4	2.0	1.6	1.5	1.2	1.7	1.5	1.8	1.5	1.9	1.5	2.4	1.9
C18:0	3.6	3.9	3.8	4.1	4.1	4.3	4.1	4.3	3.9	4.1	3.1	3.3
C18:1	47.9	51.2	49.5	52.4	47.9	50.4	47.4*	50.1	48.1*	50.9	47.7	50.6
C18:2	8.2	7.7	7.0	6.5	7.0	6.6	7.6	7.1	6.6	6.3	7.9	7.6
C18:3	10.3	8.7	9.6	8.3	10.1	8.7	10.0	8.7	10.8	9.4	11.7	9.9
C18:4	1.2	1.1	1.0	0.9	1.1	1.0	1.1	1.0	1.3	1.2	1.4	1.2
Other	1.2	0.9	1.0	0.8	1.0	0.8	1.2	0.8	1.3	0.8	0.8	1.0

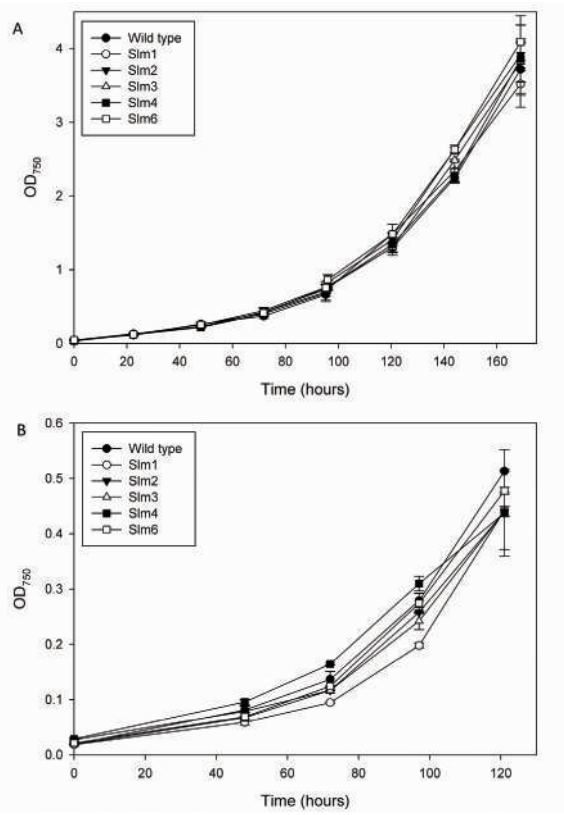

Figure 3.A1 Growth curve for starchless mutants and wild type under day night regime. Two separate experiments were carried out to study the effect of day night cycles on all strains. 16:8 hour day night regime was applied. The growth was studied by measuring the OD750 daily. (A) Cultivation in triplicate cultures for 7 days. (B) Cultivation in duplicate cultures for 5 days. Error bars indicate the distance to the mean. It can be seen that there are not many differences between the wild type and starchless mutants under these conditions.

Chapter 4

Superior triacylglycerol (TAG) accumulation in starchless mutants of *Scenedesmus obliquus* (II): evaluation of TAG yield and productivity in controlled photobioreactors

This chapter has been published as:
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Superior triacylglycerol (TAG) accumulation in starchless mutants of Scenedesmus obliquus (II): evaluation of TAG yield and productivity in controlled photobioreactors
Biotechnology for Biofuels 7:70

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Many microalgae accumulate carbohydrates simultaneously with triacylglycerol (TAG) upon nitrogen starvation, and these products compete for photosynthetic products and metabolites from the central carbon metabolism. As shown for starchless mutants of the non-oleaginous model alga *Chlamydomonas reinhardtii*, reduced carbohydrate synthesis can enhance TAG production. However, these mutants still have a lower TAG productivity than wild-type oleaginous microalgae. Recently, several starchless mutants of the oleaginous microalga *Scenedesmus obliquus* were obtained which showed improved TAG content and productivity. The most promising mutant, slm1, is compared in detail to wild-type *S. obliquus* in controlled photobioreactors. In the slm1 mutant, the maximum TAG content increased to 57±0.2% of dry weight versus 45±1% in the wild-type. In the wild-type, TAG and starch were accumulated simultaneously during initial nitrogen starvation, and starch was subsequently degraded and likely converted into TAG. The starchless mutant did not produce starch and the liberated photosynthetic capacity was directed towards TAG synthesis. This increased the maximum yield of TAG on light by 51%, from 0.144±0.004 in the wild-type to 0.217±0.011 g TAG mol^{-1} photon in the slm1 mutant. No differences in photosynthetic efficiency between the slm1 mutant and the wild-type were observed, indicating that the mutation specifically altered carbon partitioning while leaving the photosynthetic capacity unaffected. The yield of TAG on light can be improved by 51% by using the slm1 starchless mutant of *S. obliquus,* and a similar improvement seems realistic for the areal productivity in outdoor cultivation. The photosynthetic performance is not negatively affected in the slm1 and the main difference with the wild-type is an improved carbon partitioning towards TAG.

4.1 Introduction

Microalgae are well known for their ability to produce large quantities of triacylglycerol (TAG), which can be used as a resource for food, feed, and fuel production (Draaisma et al., 2013; Wijffels and Barbosa, 2010). Microalgae-derived TAGs can be competitive to oils derived from terrestrial plants due to the higher areal productivities of microalgae and because no arable land is required for their cultivation (Wijffels and Barbosa, 2010). However, the economic costs and carbon footprint of photobioreactors make it necessary to improve the areal TAG productivity even further (Brentner et al., 2011; Norsker et al., 2011).

The dogma on the physiological role of TAG synthesis is that TAG serves as a compact energy and carbon storage pool when formation of functional biomass is impaired. Furthermore, TAG can serve as an electron sink under unfavorable conditions for growth. An electron sink under these conditions prevents the build-up of photosynthetic products which would otherwise have resulted in over-reduction of the electron transport chains. Such over-reduction can result in the transfer of electrons to O_2, which results in the formation of reactive oxygen species (ROSs), such as H_2O_2, or superoxide (Asada, 2006). These ROS can cause damage to the cell. Production of TAG can thus protect the cell against the damage induced by adverse growth conditions (Hu et al., 2008). This is reflected by the fact that under nutrient replete conditions only trace amounts of TAG are produced. However, as a response to nitrogen starvation, TAG can be accumulated to over 40% of dry weight by many oleaginous microalgae species (Breuer et al., 2012; Griffiths et al., 2011; Hu et al., 2008).

Although nitrogen starvation reduces photosynthetic efficiency (Klok et al., 2013b), photosynthesis and carbon assimilation continue for a certain period when the microalgae are exposed to nitrogen depleted conditions (Simionato et al., 2013). This is supported by the observation of an up to eight-fold increase in biomass dry weight concentration after nitrogen depletion in some species (Breuer et al., 2012). This increase in biomass can be explained by de novo production of nitrogen-free storage molecules such as TAG and carbohydrates. Even in the most oleaginous species, the increase in dry weight cannot solely be explained by the observed TAG production (Breuer et al., 2012). Other storage components such as starch are simultaneously accumulated and can easily account for over 40% of the newly produced biomass (Breuer et al., 2012; Klok et al., 2013b; Li et al., 2011; Zhu et al., 2013). Diverting this large carbon flow away from carbohydrates towards TAG could substantially enhance TAG productivity (Li et al., 2010a).

The partitioning of assimilated carbon between TAG and carbohydrates during nitrogen starvation is a complex and highly regulated process as indicated by the activity of

transcription factors and the observed changes in transcriptome and proteome during nutrient starvation (Blaby et al., 2013; Boyle et al., 2012; Dong et al., 2013; Valenzuela et al., 2012). In addition, it is often proposed that the production rates of TAG and storage carbohydrates are influenced by competition for common pre-cursors, that is, intermediates of the central carbon metabolism such as glyceraldehyde-3-phosphate (GAP) or acetyl coenzyme A (acetyl-CoA) (Fan et al., 2012; Msanne et al., 2012). Modifying the activity of either pathway using strain improvement techniques could therefore potentially affect the carbon partitioning between TAG and storage carbohydrates (Li et al., 2010a). One commonly attempted strategy to accomplish this is to over-express reactions in the TAG synthesis pathway, such as the initial step in fatty acid synthesis catalyzed by acetyl-CoA carboxylase (Dunahay et al., 1996) or the acyl-transfer step catalysed by diacylglycerol acyltransferase (DGAT) (La Russa et al., 2012). However, attempts to improve TAG production by increasing the expression of genes involved in the TAG biosynthesis pathway have been mostly unsuccessful as reviewed by Li et al. (2010b).

Another commonly employed strategy is to down-regulate or inhibit the competing carbohydrate (for example, starch) synthesis. Several successful attempts have been made to enhance TAG production by reducing or eliminating starch synthesis (Li et al., 2010a; Li et al., 2010b; Ramazanov and Ramazanov, 2006; Siaut et al., 2011; Wang et al., 2009). For example, Li et al. (2010b) observed an eight-fold increase in TAG content (reaching a TAG content of 32.6% of dry weight) under mixotrophic conditions and Li et al. (2010a) observed a four-fold increase in volumetric TAG productivity under photoautotrophic conditions, both using the starchless BAFJ5 mutant of *Chlamydomonas reinhardtii*.

Although cellular TAG contents are generally enhanced in starchless or impaired mutants, the overall TAG productivity of such mutant cultures is not always improved. This is because their biomass productivity under nitrogen starvation conditions is often largely reduced or even completely impaired compared to their wild-type strains (Li et al., 2010b). This reduction in biomass productivity is often poorly characterized with the focus only directed at the TAG content, making conclusive evaluations of the mutant performance difficult. The decrease in biomass productivity could, among other possible explanations, be a result of additional mutations in, for example, the photosynthetic machinery (Siaut et al., 2011) or of insufficient capacity to channel all excess photosynthate towards TAG. Especially in the latter case, the impact of starch deficiency on oleaginous microalgae might be fundamentally different from that on non-oleaginous microalgae. Namely, a starchless oleaginous microalga might be able to redirect most of the light energy that would otherwise have been used for starch synthesis towards TAG, whereas a non-oleaginous microalga might be unable to utilize this light energy. For a non-oleaginous microalga, this could thus lead to a quick build-up of photosynthetic products, which in turn could result in over-reduction of the photosynthetic machinery and the

formation of harmful ROSs (Asada, 2006). This might occur at a lower rate in starchless oleaginous microalgae that are able to channel most excess photosynthate towards TAG synthesis.

Most previous work on starchless mutants is performed in the non-oleaginous model alga *C. reinhardtii* and shows that the use of starchless mutants can be a feasible strategy to enhance TAG production. However, even these starchless mutants under-achieve in TAG production compared to good-performing wild-type oleaginous microalgae. The TAG contents of these *C. reinhardtii* mutants are at best comparable to those of oleaginous microalgae (Breuer et al., 2012; Li et al., 2010a; Li et al., 2010b). In addition, upon nitrogen starvation their biomass productivity decreases to a much bigger extent than that of wild-type oleaginous microalgae (Breuer et al., 2012; Li et al., 2010b). Therefore, it is important to study the effect of disabling starch formation on TAG production in good-performing oleaginous microalgae.

In previous research, *Scenedesmus obliquus* (recently suggested to be reclassified to *Acutodesmus obliquus* (Krienitz and Bock, 2012)) was identified as a very promising microalga to produce TAG (Breuer et al., 2012; Griffiths et al., 2011) and recently several starchless mutants of *S. obliquus* were obtained (de Jaeger et al., 2014). In shake-flask studies, the slm1 mutant showed both an enhanced TAG content during initial nitrogen starvation and a biomass productivity comparable to that of the wild-type, resulting in a net increase in volumetric TAG productivity (de Jaeger et al., 2014). In this work, a more detailed and quantitative comparison, performed in controlled photobioreactors, of the *S. obliquus* wild-type and this starchless mutant is presented.

4.2 Methods

4.2.1 Strains, pre-culture conditions, and cultivation medium

Wild-type (wt) *S. obliquus* UTEX 393 (recently suggested to be reclassified to *Acutodesmus obliquus* (Krienitz and Bock, 2012)) was obtained from the University of Texas Culture collection of algae (UTEX). The starchless mutant (slm1) was obtained using UV radiation-induced random mutagenesis on the wild-type strain of *S. obliquus* (de Jaeger et al., 2014). The culture medium was similar to that described by Breuer et al. (2013b) with the exception that all vitamins were omitted from the culture medium. The culture medium was autotrophic and contained 10 mM KNO_3 as the limiting nutrient. All other required nutrients were present in excess. Pre-cultures were maintained in 16:8h light:dark cycles as described by Breuer et al. (2012). Both the wt and the slm1 were able to continue growing after multiple serial dilutions while being cultivated autotrophically under these day-night cycles (16:8 light:dark).

4.2.2 Experimental conditions

Batch cultivations were performed in flat-panel airlift-loop photobioreactors with a working volume of 1.7 l (Labfors 5 Lux, Infors HT, Switzerland). The reactor design is similar to that described by Klok et al. (2013a). The reactors were sparged with air containing 2% CO_2 at 1 L min^{-1}. The reactors were continuously illuminated (24h day^{-1}) using LED lamps with a warm white spectrum located on the culture side of the reactor. The incident light intensity was calibrated by measuring the average light intensity on the culture side of the front glass plate. The light path (reactor depth) was 2 cm. The temperature was controlled at 27.5°C and the pH was controlled at pH 7 using automatic addition of 1M HCl. These values for pH and temperature were found to be optimal for both growth and TAG accumulation in *S. obliquus* in previous research (Breuer et al., 2013b). A few milliliters of a 1% Antifoam B solution (J. T. Baker) were added manually when excessive foaming was visible. Prior to inoculation, reactors were heat-sterilized and subsequently filled with 0.2 µm filter-sterilized medium. Reactors were inoculated at 50 mg algae dry matter L^{-1} and grown at an incident light intensity of 100 µmol m^{-2} s^{-1} until the biomass density reached 0.3 to 1 g dry weight (DW) L^{-1} (typically after 48 h). At this point the incident light intensity was increased to 500 µmol m^{-2} s^{-1}. Periodically, samples were taken aseptically and analyzed for dry weight concentration, biomass composition, residual dissolved NO_3^-, Fv/Fm ratio, absorbance spectrum, and absorbance cross section. After nitrogen was depleted, more than 95% of the incident light was absorbed. Therefore, for simplicity, in the calculations it is assumed that the absorbed light is identical to the incident light intensity.

Although a condenser was installed at the gas exhaust of the reactor, water losses were present due to evaporation. Evaporation was quantified in a separate reactor that was filled with water and operated under the same conditions as during cultivation. The evaporation rate was 0.9 ml h^{-1}. Evaporation results in concentration of the biomass, which thus has an effect on the measured dry weight concentration. The measured biomass composition is unaffected. The biomass concentration was corrected for evaporation using a correction factor:

$$C_{x,corrected} = \frac{V_{observed}}{V_{without\ evaporation}} c_{x,observed} \qquad \text{(Eq. 4.1)}$$

The correction factor decreased from 1 at the start of the cultivation to a minimum between 0.60 and 0.71 (depending on the duration of the cultivation) at the end of the cultivation. All presented results and calculations throughout this work are based on the concentrations corrected for evaporation. Evaporation rates were similar in all experiments and therefore did not result in a biased comparison between the slm1 and wt in any way. However, evaporation and the accompanying concentration effect did increase the steepness of the light gradient in the reactor and reduced light penetration and biomass specific light absorption rates.

4.2.3 Dry weight

The dry weight concentration was determined by filtrating culture broth over pre-weighted glass fiber filters and measuring the weight increase of the filters after drying at 95°C as described by Kliphuis et al. (2012).

4.2.4 Total fatty acid

The total fatty acid (TFA) concentration was determined by a sequence of cell disruption, total lipid extraction in chloroform:methanol, trans-esterification of acyl lipids to fatty acid methyl esters (FAMEs) and quantification of FAMEs using gas chromatography as described by Breuer et al. (2013a). Tripentadecanoin was used as an internal standard.

4.2.5 Triacylglycerol

The TAG concentration was determined by separating the total lipid extract, obtained using the aforementioned method, into a TAG and polar lipid pool using a solid phase extraction column (SPE) as described by Breuer et al. (2013b), followed by transesterification and quantification of the fatty acids in the TAG pool as described by Breuer et al. (2013a).

4.2.6 Starch

The starch concentration was determined using an AA/AMG Total Starch Kit (Megazyme, Ireland) with modifications as described by de Jaeger et al. (2014). The procedure consisted of a sequence of cell disruption, starch precipitation using an aqueous solution of 80% ethanol, enzymatic hydrolysis of starch to glucose monomers using α-amylase and amyloglucosidase, and a spectrophotometric based assay for quantification of glucose monomers.

4.2.7 Dissolved nitrate

Dissolved nitrate was analyzed in supernatant using a Seal analytical AQ2 nutrient analyzer (SEAL Analytical Inc., USA) according to the manufacturer's instructions.

4.2.8 Fv/Fm

Pulse amplitude modulation (PAM) fluorometry was used to determine the Fv/Fm ratio using an AquaPen AP-100 fluorescence spectrophotometer (PSI, Czech Republic) according to the manufacturer's instructions. Cultures were diluted in demineralized water to an optical density of 0.4 at 750nm in a cuvette with a light path of 10mm (equivalent to a concentration of 0.2 g DW L^{-1}) and adapted to dark conditions for 15 minutes prior to the measurement. It was confirmed that longer dark-adaptation times did not affect the results.

4.2.9 Absorbance spectrum and absorbance cross section

Cell suspensions were diluted to an optical density at 750nm of 1.4 to 1.6 as measured in a cuvette with a 1 cm light path. Subsequently, the absorbance spectrum was measured in these diluted cell suspensions with a Shimadzu UV-2600 integrating sphere spectrophotometer in the spectrum 300 to 750nm, which results in the absorbance spectrum corrected for scattering. Residual scattering was calculated as the average absorbance between 740 and 750nm and subtracted from the absorbance spectrum. From this absorbance spectrum, the average biomass dry weight specific absorbance cross section between 400 and 700nm (α, unit: $m^2\ g^{-1}$) was calculated as:

$$\alpha = \frac{\sum_{400}^{700} abs_\lambda \frac{\ln(10)}{z}}{300\ DW} \qquad \text{(Eq. 4.2)}$$

Here, abs_λ is the absorbance at wavelength λ, z the light path of the cuvette (0.002 m), and DW the dry weight concentration ($g\ m^{-3}$).

4.3 Results and discussion

4.3.1 TAG and starch accumulation

For both the wild-type (wt) *S. obliquus* (UTEX 393) and a starchless mutant (slm1) of *S. obliquus* (de Jaeger et al., 2014), duplicate nitrogen run-out experiments were performed to investigate the difference in carbon partitioning between the wt and the slm1 under nitrogen depleted conditions (Figure 4.1). Reactors were inoculated at 50 mg DW L^{-1} and cultivated at an incident light intensity of 100 µmol $m^{-2}\ s^{-1}$ until the biomass concentration was 0.3-1 g DW L^{-1}, after which the incident light intensity was increased to 500 µmol $m^{-2}\ s^{-1}$. The moment of inoculation is considered as t=0.

Nitrogen was depleted from the culture medium at a biomass concentration of approximately 1.5-2 g L^{-1} and occurred 70 to 100h after inoculation (Figure 4.1). After nitrogen was depleted, carbon assimilation and biomass formation continued, mainly as a result of accumulation of TAG (both the wt and the slm1) and starch (the wt only), which is consistent with previous observations (Breuer et al., 2012; Breuer et al., 2013b; de Jaeger et al., 2014). The wt increased more rapidly in biomass concentration than the slm1 during initial nitrogen starvation and also achieved a higher maximum biomass concentration (Figure 4.1A,B).

In the wt cultivation, starch and TAG were accumulated simultaneously after nitrogen was depleted. Initially starch was produced at a much higher rate than TAG (Figure 4.1C) but when nitrogen starvation progressed starch synthesis stopped. Starch reached a maximum content of 38±2% (average of duplicate cultivations ± deviation of duplicates

from average) of dry weight after 168±2 h and a maximum concentration of 3.6±0.2 g L^{-1} after 223±10 h. Subsequently starch was degraded. The starch concentration at the end of the cultivation decreased to 0.5 g L^{-1} (6% of dry weight). During this period TAG synthesis continued and the TAG content reached a maximum of 45±1% of dry weight (4.5±0.1 g L^{-1}) (Figure 4.1C; Figure 4.2A). The simultaneous degradation of starch and production of TAG in the wt could indicate that degradation products of starch are used for the synthesis of TAG. This inter-conversion has also been suggested previously for *Pseudochlorococcum* sp. (Li et al., 2011), *C. reinhardtii* (Msanne et al., 2012), *Coccomyxa* sp. (Msanne et al., 2012), and *Chlorella zofingiensis* (also known as *Chromochloris zofingiensis*) (Zhu et

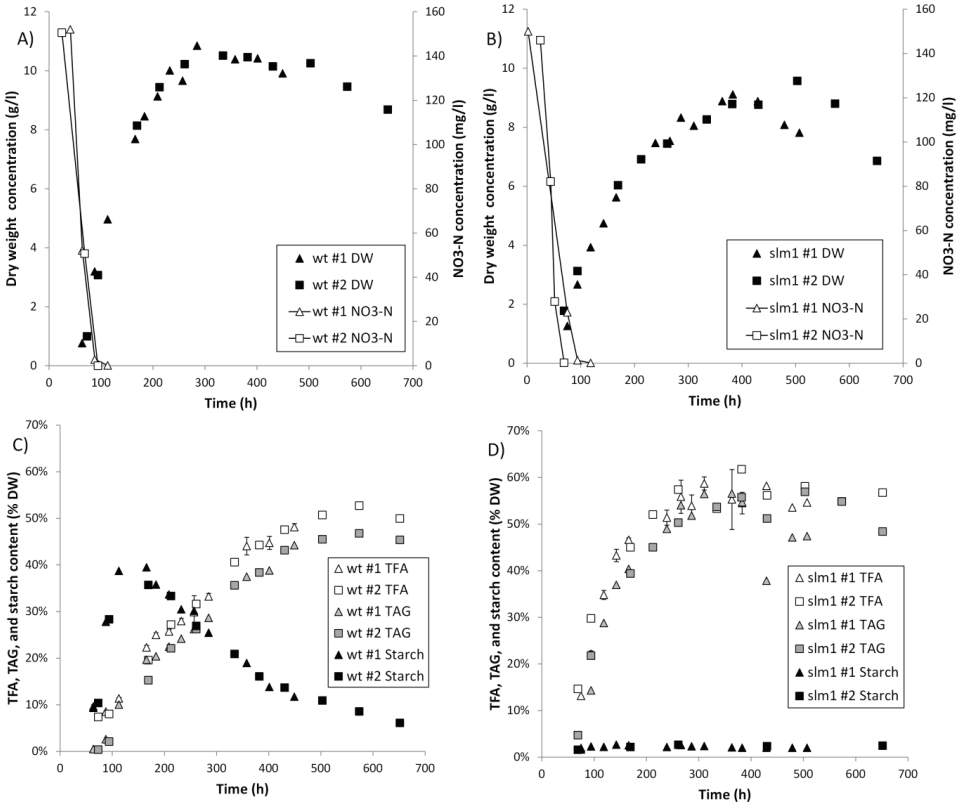

Figure 4.1 Duplicate batch nitrogen run-out cultivations of the wt and the slm1 *S. obliquus*. Left: wild-type. Right: slm1. Top: biomass concentration (g DW/l) and dissolved NO3-N concentration. Bottom: total fatty acid (TFA), TAG, and starch content. As indicated in the legend boxes in the figure, in the top figure, the black symbols represent the dry weight concentration and the open symbols represent the NO3-N concentration. In the bottom figures, the open symbols represent the total fatty acid content (TFA), the gray symbols represent the TAG content, and the black symbols represent the starch content. The results indicated with #1 and #2 in the figure legend represent the replicate cultivations.

al., 2013), as well as for conversion of chrysolaminarin into TAG in the diatom *Cyclotella cryptica* (Roessler, 1988).

In the slm1, the production of starch is negligible. As a result, the TAG content increases more rapidly in the slm1 than in the wt during initial nitrogen starvation; the TAG content in the slm1 reached a maximum of 57±0.2% of dry weight (5.2±0.2 g L^{-1}) after 433±70 h (Figure 4.1C,D; Figure 4.2A).

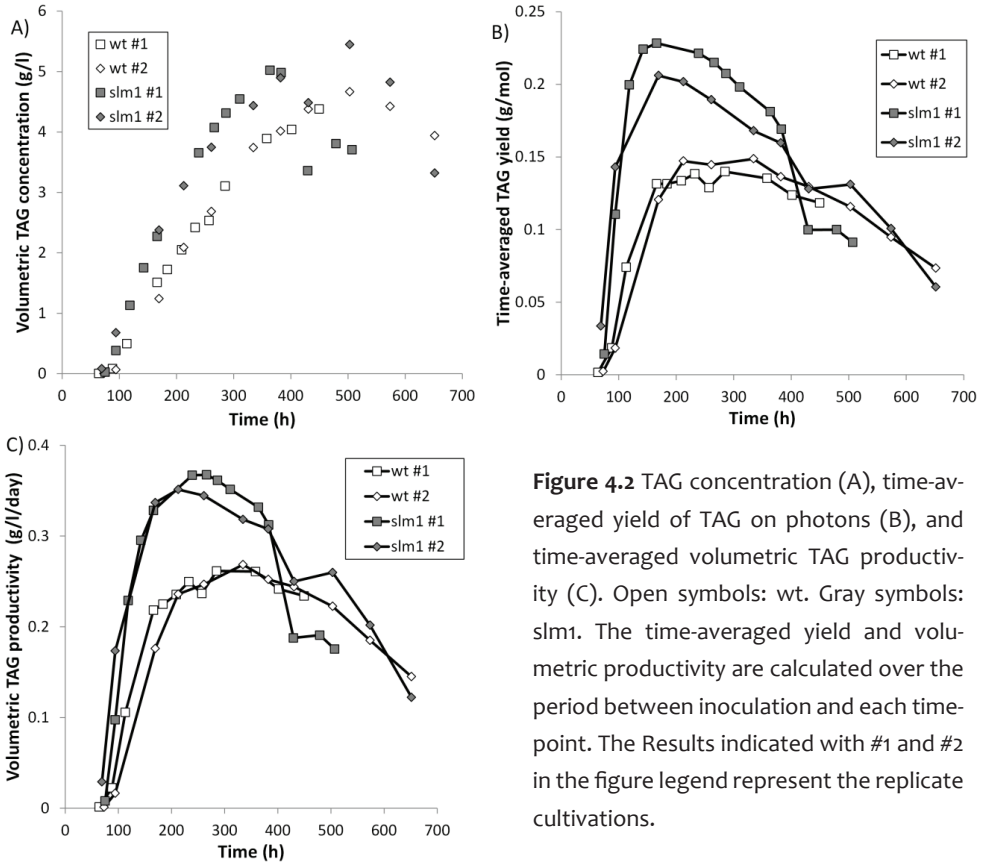

Figure 4.2 TAG concentration (A), time-averaged yield of TAG on photons (B), and time-averaged volumetric TAG productivity (C). Open symbols: wt. Gray symbols: slm1. The time-averaged yield and volumetric productivity are calculated over the period between inoculation and each time-point. The Results indicated with #1 and #2 in the figure legend represent the replicate cultivations.

In neither the wt nor the slm1 can the combined accumulation of starch and TAG completely account for the increase in dry weight after nitrogen depletion. This difference between the measured biomass constituents and dry weight concentration is relatively constant and accounts for approximately 20 to 30% of dry weight during the entire cultivation for both the wt and the slm1. Proteins are most likely not part of this residual biomass as no nitrogen source is available for protein synthesis; also protein synthesis out of non-protein nitrogen present in the biomass can only contribute very little, because

this fraction of non-protein nitrogen in the biomass is very small (Becker, 2007). It is likely that the cell wall will account for a substantial part of this residual biomass. Although little is known about the cell wall composition of *S. obliquus* and other microalgae, it is hypothesized that this residual biomass consists largely of carbohydrates (other than starch) such as cellulose, which is known to be a major constituent of the cell wall of *S. obliquus* and other microalgae (Aguirre and Bassi, 2013; Burczyk et al., 1970).

4.3.2 Yields, productivity and implications for large-scale production

Using the measured TAG concentration at each time point (Figure 4.2A) and the amount of light supplied specific to the reactor volume (calculated as the incident light intensity multiplied by the area-to-volume ratio of the reactor), the time-averaged yield of TAG on photons was calculated for each time-point (the yield of TAG on light achieved over the period between inoculation and each time point) (Figure 4.2B). Because almost no TAG is produced during nitrogen replete conditions, this yield of TAG on light is very low during the initial part of the cultivation. After nitrogen depletion, the time-averaged yield increases to a maximum of 0.217±0.011 and 0.144±0.004 g TAG mol^{-1} photon for the slm1 and wt, respectively (Figure 4.2B). This illustrates that the slm1 can achieve a 51% higher time-averaged yield of TAG on light than the wt. Similarly, the maximum volumetric productivity, calculated between inoculation and each time-point, was enhanced in the slm1 by 35% compared to the wt and increased from a maximum of 0.265±0.004 in the wt to a maximum of 0.359±0.008 g TAG l^{-1} day^{-1} in the slm1 (Figure 4.2C). During the period that these maxima in yield and volumetric productivity were maintained, the TAG content increased to over 40% of dry weight for both the wt and the slm1 (Figure 4.1C,D; Figure 4.2B,C).

After these maxima in yield and volumetric productivity were achieved, the difference in performance of the wt and the slm1 became smaller when the cultivation progressed. This can be explained by the degradation of starch in the wt and possible inter-conversion into TAG. This could enhance the TAG contents in the wt at the end of the cultivation, resulting in a smaller difference between the slm1 and wt at the end of the cultivation.

Due to the different behavior of the wt and slm1, there is a difference in the biomass concentration in the wt and slm1 cultivation (Figure 4.1). This did not result in a difference in light absorption rates between the wt and slm1 because nearly all light was absorbed in all cultures; therefore, a difference in the biomass concentration or pigmentation will only result in a difference in the light gradient in the photobioreactor. Furthermore, because all cultures were provided with the same amount of NO_3, the amount of light absorbed per N-mol and per amount of catalytic biomass (assuming that the amount of catalytic biomass is proportional to the amount of nitrogen) is exactly the same.

When algae are cultivated using sunlight, the amount of light that can be provided to the photobioreactor is limited to the insolation to that area. The maximum areal productivity is therefore directly proportional to the yield on light that can be achieved. Maximizing this yield of TAG on light can therefore contribute to improving the areal productivity of microalgal TAG production. The time-point where the highest time-averaged yield of TAG on light is achieved is therefore proposed as the optimum time-point to harvest the culture. Previously it has been shown that this yield can be enhanced by improving the photobioreactor design (Cuaresma et al., 2011; Wijffels and Barbosa, 2010) as well as optimizing cultivation conditions (Breuer et al., 2013b). In this work it is shown that this yield on light can be improved by 51% by using a starchless mutant, and a similar improvement seems realistic for the areal productivity in outdoor cultivation. It should be noted that at the moment this maximum was reached, the TAG content was over 40% of the dry weight.

In this work, all cultivations were performed using continuous illumination. However, during day-night cycles starch contents in microalgae oscillate, and starch can likely provide energy for nocturnal respiration (de Winter et al., 2013; Ral et al., 2006). This might complicate cultivation of starchless mutants in day-night cycles. In higher plants such as *Arabidopsis thaliana* it is indeed reported that starchless mutants show decreased growth rates and decreased net photosynthesis rates when grown under day-night cycles, whereas these are indistinguishable from their wild-types during continuous illumination (Caspar et al., 1985; Radakovits et al., 2010). The slm1 mutant, however, does not show decreased growth under day-night cycles under nitrogen replete conditions and possibly the role of starch can be taken over by other storage metabolites (de Jaeger et al., 2014). Further investigation of the behavior of slm1 under day-night cycles and nitrogen depleted conditions would be of future interest.

4.3.3 Photosynthetic energy distribution in the wt compared to the slm1

The biomass productivity was lower in the slm1 than in the wt during the initial period of nitrogen starvation. Because exactly the same amount of light was supplied, this might at first suggest a reduced photosynthetic efficiency in the slm1. However, lipids (for example TAG) are much more energy dense than carbohydrates, (37.6 kJ g^{-1} for lipids compared to 15.7 kJ g^{-1} for carbohydrates (Jakob et al., 2007)). The difference in metabolic costs required to produce TAG and starch can completely explain the observed difference in biomass productivity. To illustrate this, we compare the photosynthetic requirement for the observed biomass production after nitrogen depletion in the wt and the slm1. To calculate this photosynthetic requirement, it is assumed that after nitrogen depletion only TAG, starch, and other carbohydrates (such as cell wall cellulose) are produced. The TAG and starch concentration are measured at each time-point (Figure 4.1) and it is assumed that the remaining newly produced biomass consists of other carbo-

hydrates (calculated as the amount of dry weight produced minus the amounts of TAG and starch produced). The photosynthetic requirement to produce the biomass that is made between nitrogen depletion and time-point t can then be calculated by summing the quotients of the measured concentration of each biomass constituent at time-point t and the photosynthetic yield of that biomass constituent:

$$Photosynthetic\ requirement\ (t) = \frac{C_{TAG(t)}}{Y_{TAG,\ light}} + \frac{C_{Starch(t)}}{Y_{Starch,\ light}} + \frac{C_{Carbohydrate(t)}}{Y_{Carbohydrate,\ light}} \quad \text{(Eq. 4.3)}$$

In Eq. 4.3, $C_i(t)$ represents the concentration of component i (g L^{-1}) at time-point t and $Y_{i,light}$ represents the photosynthetic yield of component i (g product/mol photon). These photosynthetic yields are estimated to be 1.02 g TAG/mol photon, 3.24 g starch/mol photon, and 3.24 g carbohydrate/mol photon (see Appendix A).

Using this calculation, it appears that although the slm1 has a lower biomass productivity, the minimum photosynthetic requirement to produce that biomass is similar (Figure 4.3). This indicates that the slm1 does not have a reduced photosynthetic efficiency, but only seems to differ from the wt in terms of carbon partitioning.

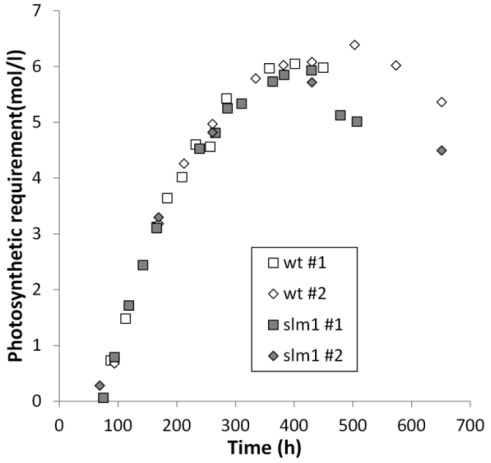

Figure 4.3 Theoretical minimum amount of photons required to produce the observed biomass after nitrogen is depleted. Open symbols: wt. Gray symbols: slm1. The results indicated with #1 and #2 in the figure legend represent the replicate cultivations.

In the wt, the calculated photosynthetic requirement also increases at the end of the cultivation, where no substantial increase in dry weight concentration is observed. This can be explained by an increase in energy density of the biomass due to a change in biomass composition (increase in TAG and decrease in starch content). This requires additional energy, which is provided by photosynthesis.

In these calculations it was assumed that the residual biomass (difference between the produced dry weight and the measured amounts of TAG and starch) consists for a large part of cell wall material that is made of carbohydrates such as cellulose (Burczyk et al., 1970). If this were a different biomass constituent with a different photosynthetic yield than carbohydrates, it would affect the calculated photosynthetic requirement. However, the estimated amount of this remaining fraction is similar in the wt and the slm1. Therefore, this will not result in a biased comparison.

It is observed in the wt that starch is first produced and subsequently degraded (Figure 4.1). This turnover is not taken into account in these calculations. However, the turnover of starch (synthesis of starch out of GAP and subsequent degradation of starch into GAP) only costs 1 ATP per glucose monomer and would only result in a minor change in the calculated photosynthetic requirement.

4.3.4 Photosynthesis

The pigmentation of the cell determines the amount of light that can be absorbed and ultimately be used for photosynthesis. The absorbance cross section of the biomass was measured and used as a proxy for the pigmentation. An up to eight-fold decrease in the biomass specific absorbance cross section (m^2 g^{-1} DW) was observed at the end of the cultivation compared to the point before nitrogen depletion in both the wt and the slm1 (Figure 4.4B). A decrease in pigmentation during nitrogen starvation is commonly observed in microalgae (Geider et al., 1998). The volumetric absorbance cross section (m^2 L^{-1}), however, remained more or less constant throughout the entire experiment. This suggests that the decrease in biomass specific absorbance cross section is mainly a result of dilution of pigments over newly formed biomass and is likely caused to a lesser extent by net degradation of pigments. In addition to a change in absorbance cross section, the absorbance spectrum, and thus the pigment class composition, changed drastically (Figure 4.4C). Photo-protective pigments (carotenoids) can be produced in response to physiological stress to prevent photo-oxidative damage (Geider et al., 1998; Lamers et al., 2008). The ratio of chlorophyll over carotenoids decreased during nitrogen starvation as is apparent from the increase in absorbance at 483nm (the observed absorbance maximum of carotenoids) relative to the absorbance at 680nm (the observed absorbance maximum of chlorophyll) (Figure 4.4D). The decrease in absorbance cross section between the slm1 and wt was similar but the slm1 showed a higher ratio of absorbance at 483nm/680nm as the nitrogen starvation progressed. This suggests that the slm1 has relatively more carotenoids than the wt. This difference between the slm1 and wt became more apparent when nitrogen starvation progressed (Figure 4.4D). These observations suggest that a progressively smaller fraction of the absorbed light is available for photosynthesis when nitrogen starvation progresses due to an increased carotenoid/chlorophyll ratio.

The variable fluorescence/maximum fluorescence ratio (Fv/Fm) was measured and can be used as a proxy for the intrinsic (or maximum) PSII quantum yield (Li et al., 2010b; Simionato et al., 2013). Although it does not directly reflect the photosynthetic efficiency achieved in the photobioreactor, it is often used as a diagnostic value for the photosynthetic performance (Maxwell and Johnson, 2000). Immediately after inoculation, the Fv/Fm ratio started substantially below the maximum value that was observed (Figure 4.4A). This could possibly be due to a shock in biomass concentration and light intensity as a result of inoculation. During the nitrogen replete growth phase, the Fv/Fm ratio

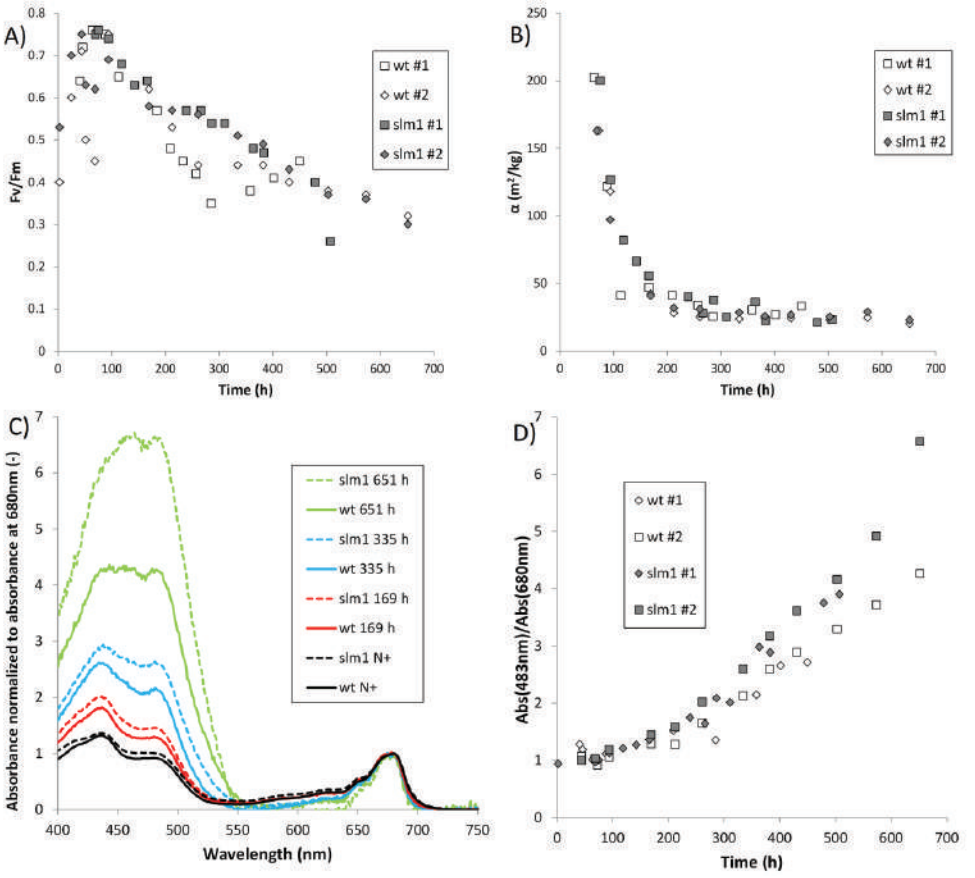

Figure 4.4 Impact of nitrogen starvation on photosynthesis. A: Fv/Fm ratio. B: Absorbance cross section. C: Absorbance spectrum of the slm1 and wt under nitrogen replete and nitrogen depleted conditions, normalized to the absorbance at 680nm. D: Ratio of absorbance at 483nm and 680nm. In figure C, the time indicated in the figure legend represents the time after inoculation. Nitrogen starvation commenced 70 to 100 h after inoculation. The spectrum that is indicated with N+ in the figure legend represents the spectrum before nitrogen was depleted. Open symbols indicate the wt and gray symbols indicate the slm1. The results indicated with #1 and #2 in the figure legend represent the replicate cultivations

increased gradually to a maximum of 0.78, which is consistent with maximum values observed in other studies (Li et al., 2010b). Once nitrogen was depleted, the Fv/Fm ratio gradually decreased, as is commonly observed (Li et al., 2010b; Simionato et al., 2013). This could be an indication of increased damage to the photosystems. Fv/Fm ratios in the slm1 are comparable to, or even slightly higher than the Fv/Fm ratios in the wt. This is consistent with the observation presented in Figure 4.3, that the photosynthetic performance is not negatively affected in the slm1 and that the main difference with the wt is an improved carbon partitioning towards TAG in the slm1.

4.4 Conclusions

The maximum TAG content increased from 45 ± 1% in wild-type to 57 ± 0.2% of dry weight in starchless *S. obliquus* (slm1). The slm1 had a lower biomass productivity, which can completely be explained by the higher energy requirement to produce TAG compared to starch. The maximum yield of TAG on light in the mutant increased from 0.144±0.004 to 0.217±0.011 g TAG/mol photon, and a 51% improvement in areal TAG productivity therefore seems realistic for outdoor cultivation. This work highlights the potential of improved carbon partitioning using a starchless mutant to increase TAG productivity in oleaginous microalgae.

Acknowledgements

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Appendix

TAG consists of a glycerol backbone with three fatty acids. Because approximately 50% of the fatty acids in TAG are oleic acid molecules in *S. obliquus* (Breuer et al., 2013b), triolein is used as the reference TAG molecule to calculate the photosynthetic yield of TAG. In the production of one molecule of oleic acid from CO_2, 70 molecules of NADPH and 71 molecules of ATP are required and 18 molecules of NADH are produced. In the production of the glycerol backbone 7 NADPH and 9 ATP are consumed (Johnson and Alric, 2013). Finally, in the condensation of the fatty acids to the glycerol backbone, it is assumed that 1 ATP is consumed for each fatty acid. This results in the net utilization of cofactors as presented in Eq. 4.A1.

$$217\ NADPH + 225\ ATP + 54\ NAD^+ \longrightarrow 54\ NADH + 1\ triolein + 217\ NADP^+ + 225\ ADP + 225\ P_i \qquad \text{(Eq. 4.A1)}$$

Similarly, one glucose monomer of starch can be produced out of 19 ATP and 12 NADPH (Eq. 4.A2). It is assumed that production of other carbohydrates (such as cell wall cellulose) is similar to production of starch in terms of metabolic requirements.

$$12\ NADPH + 19\ ATP \longrightarrow 1\ starch\ glucose\ monomer + 12\ NADP^+ + 19\ ADP + 19\ P_i \qquad \text{(Eq. 4.A1)}$$

The required cofactors can be provided using photosynthesis through either linear electron transport (theoretical maximum: 8 photons ⟶ 3 ATP + 2 NADPH) or cyclic electron transport (theoretical maximum: 2 photons ⟶ 1 ATP). Furthermore, it is assumed that oxidative phosphorylation can be used to provide 2.5 ATP using 1 NADH.

Using these stoichiometric relationships, it can be calculated that a minimum of 868 photons are required to produce 1 mol of triolein and a minimum of 50 photons are required to produce 1 mol of starch glucose monomers. Using the molecular weights of triolein (885 g mol^{-1}) and starch glucose monomers (162 g mol^{-1}), theoretical maximum yields of 1.02 g TAG mol^{-1} photon and 3.24 g starch/mol photon can be found.

Note that according to the stoichiometric relationship of triolein production, additional NADH is produced (Eq. A1). If this NADH can be used to reduce $NADP^+$ to NADPH, the yield of TAG on photons could increase to 1.36 g TAG/mol photon.

Chapter 5

Superior triacylglycerol (TAG) accumulation in starchless mutant of *Scenedesmus obliquus* is caused by a SNP in the small subunit of ADP-glucose pyrophosphorylase

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The productivity of lipids in microalgae needs to be increased to make microalgal derived oils economically feasible. The lipid productivity can be increased by obstructing the starch biosynthesis pathway, enabling the carbon to be redirected towards triacylglycerol. In this study the mutation responsible for the starchless mutant Slm1 phenotype was identified and the starch biosynthesis genes were studied at the transcriptional level. A single nucleotide polymorphism was discovered in the small subunit of the starch biosynthesis rate-controlling enzyme ADP-glucose pyrophosphorylase. A transversion caused by UV mutagenesis resulted in the introduction of a STOP codon in the messenger RNA of the enzyme. The mutation was confirmed by sequencing the PCR product of the small subunit of the ADP-glucose pyrophosphorylase gene in the wild type and Slm1 strains. The characterization of the mutation increases the understanding of carbon partitioning in oleaginous microalgae, leading to a promising target for future genetic engineering approaches to increase triacylglycerol accumulation in microalgae.

5.1 Introduction

Economically feasible production of microalgal oils is only possible if the lipid productivity in microalgae is improved. The lipid productivity can be increased significantly by rerouting carbon partitioning towards triacylglycerol (TAG) production instead of carbohydrates. The main storage carbohydrate in most microalgae is starch, and by reducing the flux through the starch pathway the TAG content can be increased. This concept has been proven in the predominantly starch accumulating microalgae *Chlamydomonas reinhardtii* (Li et al., 2010a; Zabawinski et al., 2001). The starchless mutants generated in *C. reinhardtii* do over accumulate TAG, but have a much lower biomass productivity, especially under day/night light regimes. This is due to the fact that starch is normally dissimilated at night to generate carbon and energy. Recently the starch biosynthetic pathway has been knocked out in the oleaginous microalgae *Scenedesmus obliquus* (de Jaeger et al., 2014) using UV mutagenesis. The TAG productivity was strongly enhanced in starchless mutants of *S. obliquus*, without compromising growth and robustness of the strain (Breuer et al., 2014; de Jaeger et al., 2014). The starchless mutant with the highest TAG yield and productivity is the starchless mutant 1 (Slm1). Slm1 showed an increase in maximal TAG yield on light of 51%, and the volumetric productivity was 35% higher than the wild type strain (Breuer et al., 2014). The exact regulatory mechanism that results in the increased TAG productivity in Slm1 is not completely clear. It is known that the mutant does not make starch, but there is no knowledge of the specific mutations and in which genes they occurred. The aim of this study was to find the disrupted gene(s) and study the influence of their disruption on carbon metabolism using mRNA sequencing. This was done by sequencing the mRNA of both the wild type and Slm1 strains of *S. obliquus* under nitrogen replete conditions.

5.2 Material and Methods

5.2.1 Strains, medium and pre-culture conditions

Scenedesmus obliquus UTEX 393 wild type (University of Texas, Austin, USA) and starchless mutant 1 (Slm1) (de Jaeger et al., 2014) were maintained in 100 ml filter sterilized fresh water medium in 250 ml Erlenmeyer shake flasks at 50 µmol s^{-1} m^{-2} at 25°C and shaken at 125 rpm. The fresh water medium consisted of: KNO_3 50.5 mM; Na_2SO_4 4.6 mM; HEPES 100 mM; $MgSO_4.7H_2O$ 1 mM; CaCl2.2H2O 0.5 mM; K2HPO4 4.1 mM; NaHCO3 10 mM; NaFeEDTA 0.14 mM; Na2.EDTA.2H2O 0.4 mM; MnCl2.4H2O 96 µM; ZnSO4.7H2O 21 µM; CoCl2.6H2O 6 µM; CuSO4.5H2O 6.6 µM; Na2MoO4.2H2O 0.5 µM. The pH was set to pH 7.5 using NaOH and buffered with 100 mM HEPES. The medium was filter sterilized (0.2 µm) prior to use.

5.2.2 Experimental set up

Strains were cultivated in 100 ml Fresh water medium in 300 ml Erlenmeyer flasks in continuous light (100 µmol s^{-1} m^{-2}) at 25°C and shaken at 125 rpm and enriched with 2.5% CO_2. Each strain was cultivated in triplicate and inoculated at OD_{750} around 1.5. After 48 hours the culture (OD_{750} around 4) was sampled to analyze cell dry weight (CDW), total fatty acid (TFA), starch, and RNA.

5.2.3 Cell dry weight concentration

CDW concentrations were determined on biological replicates. Around 1.5 mg of biomass was filtered through pre-dried (100°C overnight) and pre-weight Whatman glass fiber filter paper (GF/F; Whatman International Ltd, Maidstone, UK). The filter was washed with 50 ml of filtered demineralized water supplemented with an equimolar concentration of NH_4HCO_2 to prevent osmotic shock and subsequently dried overnight at 100°C before weighing.

5.2.4 Starch analysis

The starch content was analyzed using the total Starch assay (Megazyme International, Wicklow, Ireland) following the protocol described previously (de Jaeger et al., 2014). The method is based on enzymatic degradation of starch to glucose monomers by α-amylase and amyloglucosidase enzymes and measuring glucose monomers in a spectrophotometric-based assay for quantification against a D-glucose calibration control series at a wavelength of 510 nm.

5.2.5 Total fatty acid analysis

TFA extraction and quantification was executed as described by Breuer *et al.* (Breuer et al., 2013a) with the following adjustments. Around 5 mg of pellet was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) and lyophilized overnight. Freeze-dried cells were disrupted by a 30 min bead beating step in the presence of a chloroform:methanol mixture (1:1.25) to extract the lipids from the biomass. Tripentadecanoin (T4257; Sigma-Aldrich, St Louis, MO, USA) internal standard was added to the extraction mixture to enable fatty acid quantification. Methylation of the fatty acids to fatty acid methyl esters (FAMEs) and the quantification of the FAMEs were performed as described by Breuer et al. (Breuer et al., 2013a).

5.2.6 RNA extraction

Samples for RNA isolation were immediately processed after sampling and kept on ice. Cells were collected by centrifugation at 4.000xg at 0°C for three minutes and immediately frozen in liquid nitrogen before storage at -80°C until further extraction. Cells were disrupted by grinding the pellet using mortar and pestle and liquid nitrogen. A 5 ml volume of heated (65°C) phenol-chloroform and 5 ml of extraction buffer (10 mM EDTA,

1% sodium dodecyl sulphate (SDS), 2% 2-mercaptoethaol and 200 mM sodium acetate, pH 5) was added to the ground biomass. The RNA was precipitated by addition of 1/3 volume 8 M lithium chloride (LiCl) enriched with 1% 2-mercaptoethanol. The RNA pellet was washed with 2 M LiCl and twice with 70% ethanol. After evaporation of the last residues of ethanol, the pellet was resuspended in RNase free H_2O.

5.2.7 RNA sequencing and RNAseq analysis

Quality control and sequencing was outsourced to BaseClear BV (Leiden, The Netherlands). Single-end 50bp long sequence reads were generated using the Illumina HiSeq2500 system. The FASTQ sequence reads were generated using the Illumina Casava pipeline version 1.8.3. Quality assessment was done based on Illumina Chastity filtering and adapters were removed using an in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0. The quality of the FASTQ sequences was enhanced by trimming off low-quality bases using CLC Genomics Workbench version 7.5.1. The quality-filtered sequence reads were aligned against the reference draft genome of *S. obliquus* using CLC Genomics Workbench and expression values were calculated with the "RNA-seq" option. Comparison of expression values and statistical analysis have been performed with the "Expression analysis" option. The selected expression measure is the RPKM, which is defined as the Reads per Kilobase of exon model per Million mapped reads (Mortazavi et al., 2008) and seeks to normalize for the difference in number of mapped reads between samples as well as the transcript length. Principle component analysis was performed using the RPKM values and samples are clustered into groups using a hierarchical clustering approach (Appendix figure 5.A1).

5.2.8 DNA extraction

DNA was extracted from wild type and slm1 cultures (2 g wet pellet) to demonstrate the mutation in the DNA of the STA6 gene. DNA was isolated using similar approach as for RNA extraction with the following modifications. After phenol-chloroform extraction, the DNA was precipitated by isopropanol sodium acetate. The quality of the extracted DNA was checked by agarose gel electrophoresis and quantified using a NanoDrop 1000 spectrophotometer (NanoDrop). 250 ng DNA was used in a 25 µl PCR reaction, together with 1 µl (10 µM) forward primer 5'-TGC-ACA-AGG-GCG-TTC-GTG-TG-3' and reverse primer 5'-TGG-CCA-ACG-CAG-CTC-AAT-AC-3' and 12.5 µl DreamTaq (Fermentas). The PCR product was gel purified and sequenced using the primers listed above.

5.3 Results and discussion

Since starch is accumulated under nitrogen replete conditions in wild type *S. obliquus,* the mRNA of the wild type *S. obliquus* and Slm1 strain was sequenced in triplicate under nitrogen replete conditions. This data provides the information on the mutation and the influence of the mutation on the transcription of carbon metabolism related genes.

The transcriptome analysis resulted in 13095 transcripts from which 2012 transcript could be annotated. In table 5.1 the biomass composition of the wild type and Slm1 strains are given at the time of sampling. The starch content in the Slm1 is around the detection limit of the protocol and much lower than the starch content in the wild type. The values that are found in the wild type are lower compared to previous observations, but this can be explained by the fact that the cells received less light due to the high cell density of the culture as has been observed previously in *S. obliquus* (Breuer et al., 2015).

Table 5.1 Biomass composition of wild type and Slm1 strains. The values are the average of triplicate samples and the standard deviation is given.

	DW			**OD750**			**Starch**			**TFA**		
	$g\ L^{-1}$						%CDW			%CDW		
Wild type	1.81	±	0.06	4.08	±	0.02	3.57	±	0.10	9.94	±	0.12
Slm1	1.90	±	0.06	4.35	±	0.00	0.23	±	0.03	10.54	±	0.15

The expression of the genes involved in the starch biosynthesis pathway are shown in figure 5.1. In the upper part of the glycolysis, D-Fructose-6P is converted into D-Glucose-6P by glucose-6-phosphate isomerase (EC:5.3.1.9). Next phosphoglucomutase (EC:5.4.2.2) converts D-Glucose-6P into D-Glucose-1P and subsequently ADP-glucose pyrophosphorylase (EC:2.7.7.27) catalyzes the conversion of D-Glucose-1P into ADP-glucose. From ADP-Glucose the pathway continues towards amylose using starch synthase (EC:2.4.1.21). Finally amylose is converted to starch by the glycogen branching enzyme (EC:2.4.1.18) (Figure 5.1). Starch can be degraded by α-amylase (EC:3.2.1.1), β-amylase (EC:3.2.1.2), and starch phosphorylase (EC:4.2.1.1). Not all enzymes could be annotated and lack an expression value, others have several values, which can be explained by the fact that they have several subunits for the same enzyme. All starch synthesis genes are upregulated in Slm1 compared to the wild type except for the ADP-glucose pyrophosphorylase, which is decreased fivefold (Figure 5.1). Based on this, it seems likely that this gene is mutated and therefore we focused our study on ADP-glucose pyrophosphorylase at first. Based on the expression data we expected that the mutation was present in one of the subunits of this enzyme. The Sim1 mutation leads to reduced flux through the starch biosynthetic pathway, which probably triggers overexpression of the other genes of this pathway to try to compensate for the reduced rate of starch synthesis.

Under the tested condition, the β-amylase and the starch phosphorylase genes are over-expressed in the Slm1 strain, compared to the wild type. An explanation could be that starch is a dynamic storage molecule with a high turnover, to react to limiting light conditions or in case energy is needed for cell division. The expression values of the phosphorylase enzyme should be interpreted with care, since the starch phosphorylase enzyme is poorly annotated, and could be another phosphorylase with close sequence similarity.

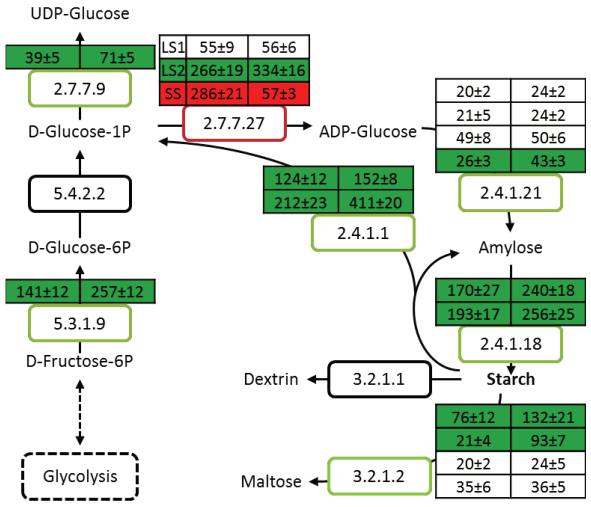

Figure 5.1 Expression of starch synthesis related genes. The values above the colored box with enzyme numbers represent the RPKM values for the wild type (left) and the Slm1 (right) strain. The values are the average of the three replicates and the standard deviation. The color of the box depicts the up (green) or down (red) regulation of the slm1 transcript compared to the wild type. When several rows of RPKM values are shown, there is more than one transcript associated with this enzyme. LS1, LS2, and SS are corresponding to large subunit 1, 2, and small subunit respectively. Enzymes EC:5.4.2.2 and EC:3.2.1.1 are not annotated.

To study the ADP-glucose pyrophosphorylase enzyme in more detail, we mapped the reads of both the wild type and slm1 strain to the native ADP-glucose pyrophosphorylase DNA sequence of *S. obliquus*. The ADP-glucose pyrophosphorylase gene consists of two large subunits and one small subunit. In *C. reinhardtii* the small and large subunits of the ADP-glucose pyrophosphorylase enzyme code for, the catalytic and regulatory subunits respectively (Stern, 2009; Zabawinski et al., 2001).

The wild type strain showed 100% sequence similarity with the nucleotide sequence of all three subunits from the previously sequenced *S. obliquus* genome (data not shown). The Slm1 reads showed a single-nucleotide polymorphism (SNP) compared to the wild type in the small subunit of ADP-glucose pyrophosphorylase. In figure 5.2 the result of the

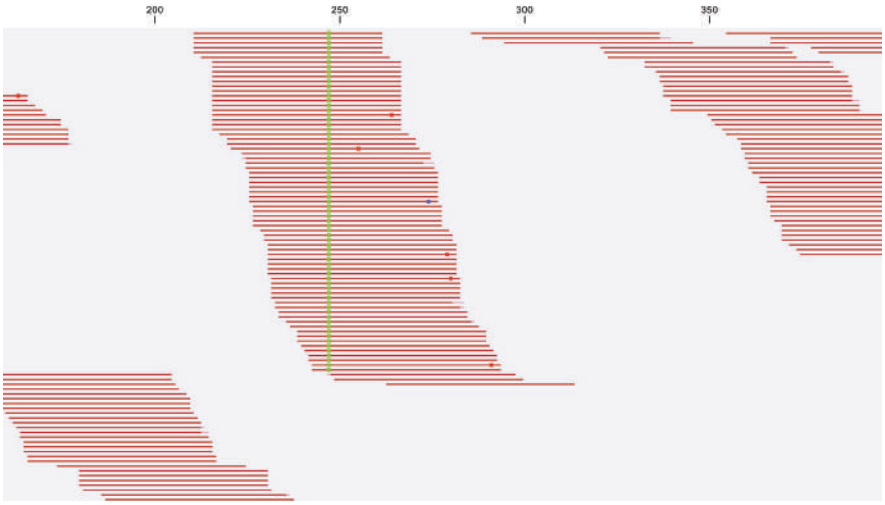

Figure 5.2 Slm1 reads mapped to the ADP-glucose pyrophosphorylase nucleotide sequence of the wild type strain. The green line represents the SNP

mapping of the Slm1 reads to the native ADP-glucose pyrophosphorylase small subunit is shown. The green line indicates the SNP in the mapped reads that have a mismatch with the nucleotide sequence. The UV mutagenesis treatment resulted in the transversion of an Adenosine (A) to a Thymine (T) at position 410 in the 3rd exon of the small subunit of ADP-glucose pyrophosphorylase gene (Appendix figure 5.A2). The SNP was confirmed by sequencing the PCR product of the ADP-glucose pyrophosphorylase small subunit. Figure 5.3A shows part of the chromatogram and the SNP is indicated by a black arrow. The SNP results in a codon change as can be seen in figure 5.3B. The wild type strain has the triplet AAG which encodes for the amino acid Lysine (K), and in the Slm1 the triplet is turned into TAG which encodes for a STOP codon. A STOP codon in the messenger RNA will signal the termination of translation by binding release factors which induces the dissociation of the ribosomal subunits, releasing the amino acid chain. In this case the STOP codon is in the middle of the mRNA sequence and will terminate the amino acid chain prematurely, causing a non-functional D-glucose pyrophosphorylase enzyme.

In storage polysaccharide biosynthesis, such as starch, ADP-glucose is used as a nucleotide sugar donor, and the ADP-glucose pyrophosphorylase catalyzes the formation of the glucosyl nucleotide from ATP and glucose-1-phosphate, and is the major rate-controlling step (Preiss and Romeo, 1994; Smith-White and Preiss, 1992). The ADP-glucose pyrophosphorylase can be activated by 3-phosphoglyceric acid (3-PGA) and inhibited by orthophosphate (Smith-White and Preiss, 1992), which couples starch biosynthesis to photosynthesis.

Although the UV mutagenesis method was applied in such a way that there is one mutation per genome on average, it is possible that several SNPs were introduced in the gDNA of the Slm1 strain. No SNPs were observed in the other starch synthesis related genes in both the wild type and Slm1 strain (data not shown), indicating that the knockout of the ADP-glucose pyrophosphorylase gene is causing the starchless, TAG over accumulating phenotype in the Slm1 strain.

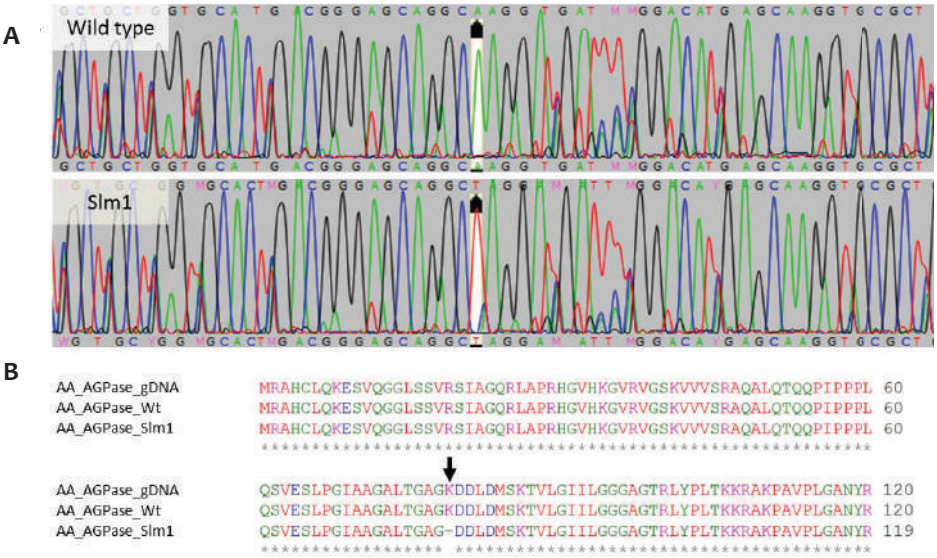

Figure 5.3 Chromatogram showing the nucleotide sequence of the wild type and Slm1 strain of the ADP-glucose pyrophosphorylase small subunit gene, obtained by sequencing the PCR product (A). The amino acid sequence obtained from the alignment of the reads to the native ADP-glucose pyrophosphorylase small subunit gene (AGPase) from the draft genome. The three lines that are shown represent the amino acid (AA) sequence of the native gene (draft genome), the wild type (PCR), and the Slm1 (PCR) strain.

The best performing starchless mutant in *C. reinhardtii* is the BAFJ5 strain obtained by Zabawinski et al., which also has a knocked-out ADP-glucose pyrophosphorylase (Zabawinski et al., 2001). This enzyme is therefore an interesting target for molecular geneticists to knock out in other species in the future. This study resulted in the characterization of the mutation in the genome of the Slm1 strain of *S. obliquus*, but there is much more to explore in this interesting improved oleaginous microalgae. It would be very interesting to study the gene expression of the wild type in comparison to the Slm1 strain under nitrogen limiting conditions, because TAG and starch accumulation are observed under these conditions. The influence of the knockdown of the starch biosynthesis pathway on the total carbon metabolism pathway would be interesting to investigate as well and should be explored in the future.

5.4 Conclusion

We identified the mutation that causes the starchless phenotype in the *S. obliquus* Slm1 strain using RNA sequencing of the wild type and Slm1 strains. In the Slm1, the rate controlling enzyme ADP-glucose pyrophosphorylase contains a single nucleotide polymorphism, which introduces a STOP codon in the 3rd exon of the catalytic small subunit of ADP-glucose pyrophosphorylase. The UV mutagenesis treatment resulted in the transversion of an Adenosine (A) to a Thymine (T), and the mutation was confirmed by sequencing the PCR product of the ADP-glucose pyrophosphorylase small subunit. The biosynthesis pathway towards starch is blocked and the excess sugar molecules are redirected towards different storage molecules, such as other carbohydrates. The influence of the starch biosynthesis pathway knockdown should be explored in more detail in the future, especially under nitrogen depleted, TAG accumulating, conditions. The small subunit of ADP-glucose pyrophosphorylase is a promising target to knock out to increase the TAG content in other oleaginous microalgae.

Acknowledgements

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Appendix

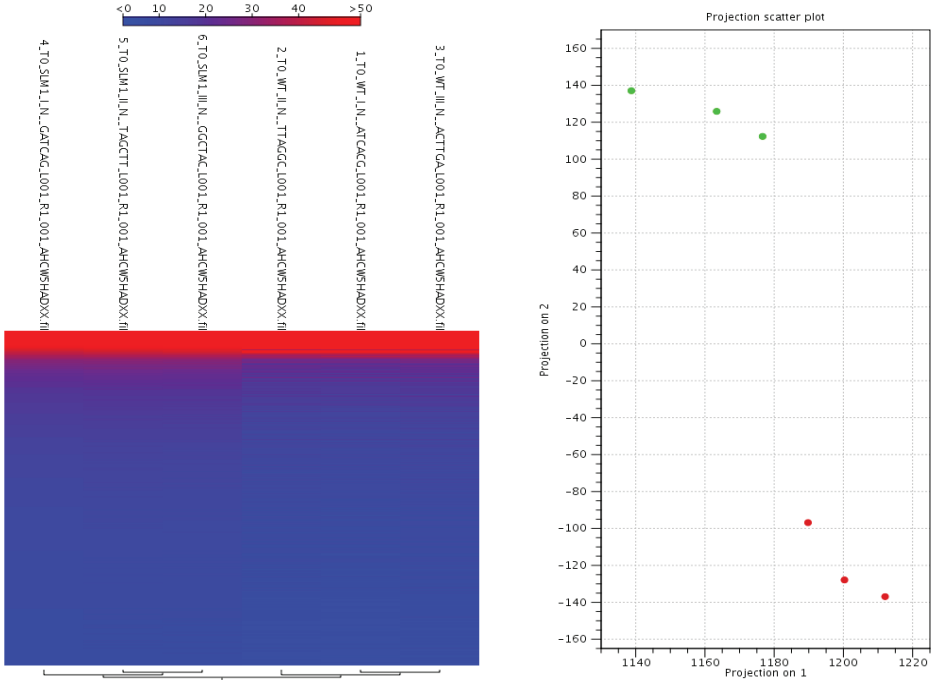

Figure 5.A1 (Left) All samples are clustered into groups using a hierarchical clustering approach and shown as a heat map for all expressed transcripts. (Right) Principle component analysis.

ATGCCAGTAGCCATGCGGGCGCATTGCCTTCAAAAAGAGTCTGTTCAGGgtgcgtttaatacatatagtcagcgttgcatgtagtttcccttgcgggggtctgttcgggag
ctcatgtacttgcacaatctgcacagGTGGTCTGAGCTCTGTGCGCTCGATCGCTGGCCAGCGCCTGGCTCCAAGGCATGGTGTGCACAAGGGCGTTCGTGTGG
GTTCAAAGgtgcgcaatcagctcccaggcgtgtcaggcacctggcttgatcgggcactgagcctggctctcccctcttcgcagGTGGTCGTGAGCAGGGCGCAGGCACTGCA
GACGCAGCAGCCTATCCCTCCTCCTCTGCAATCCGTTGAGTCACTGCCTGGGATTGCTGCTGGTGCACTGACGGGAGCAGGCAAGGATGATTTGGACA
TGAGCAAGgtgcgctgcgagttcagctctggacaaagcatctaggccgctggagggctcgcagtgtatgtgtcgatatagccatgggtattgagctgcgttggccaatctggtcagcct
gaattggaagtacggccagtgcagcactgcagttcagcgcctaatgcagtaaccgggatcttcgcctgatagaatcaattcagggcctgatcacggccttgtatgcaatgagcagcaatact
ttgacagttacaccctgctgcagcatctggtccccatccatgcaaggggattatcagccatgacgcctggcatgctgctgcatcgttgctctgctggcaaccattgcagtcggctaggccatc
cacagcagtagacagcaggagccacggccgtggccaccaacagcagaagtccagccccgcagtggttttctgaactttctgattgcaagccaacctcactgccaatgtaacatgctgccgg
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CGGTGCCGCTGGGCGCCAACTACCGCCTCATCGACATCCCCGTGTCCAACTGCGTGAACAGCAACGTGACCAAGATCTACTGCCTCACGCAGTTCAAC
TCCGCGTCGCTCAACAGGCACCTGTCGCAGGCATACgtgagtgcctcgcagcagcagcagcagcagcagcactgtggcaggttgcagggcacagtgcgggtgtggtct
ggcagaggaatcaagcacagctgtgcggcctgcatgcagctgccttgcgtttgtcgcaggctcttacaagcacaatattgcatcgccactgactaaatgaaggcactccagcagccaggcc
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nnncagcagcagcagcagcacagggcgggggctgggcagtggagtgctagtgtgtggcgtttggtggacagttgtcagcaatccatgattgtcagcatcatcatgcgcaatcgtaggat
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ccacacgtatcactcgagcacctttgttgttgcacctgcctgctggcacccgccgccaccacctgaccaggcgaccacctgtaccgcatcgatcacaaggattttgtgcgcaagcacccatat
gttgttgcaactgcctgctggccgcgctgctgctgtcagGCGACCACCTGTACCGCATGGACTACAAGGAGTTTGTGCGCAAGCACCGCGAGAGCGGCGCAGCCA
TCACCATTGCGGCGCTGCCCTGCGACGAGAAGCGCGCCAGCGCGTTCGGCCTCATGAAGATTGACGATGAGGGCCGGGTGATTGAGTTTGCTGAGAA
GCCGAGCGGTGACGCGCTCAAGGCGATGCAGGTGGACACCACCGTGCTGGGCGTGGACGCGAAGACgtgagcgggctgctgctgctgctggcacagctgct
ttgctttttttactgttttggatgcttttgcagcagtttggttaggcagcagctgctgtgtatcttcgccaacgtcgacagtgctgggcagggttgtggctcggctggcatcagcctgggttcaat
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cagCGCTGCTGAGAAGCCGTATATTGCGTCCATGGGCATCTACGTGATGACCGCCAACGTGCTCAAGGAGCTGCTGGAGAACCACATGCCCACAGCCA
ACGACTTTGGCAATGAGGTCATCCCTGAGGCGCGCAAGATGGGCTACCCGGTGCAGgtgcgttgcgagaggggcgggatgcagcaagggtcggttgcagggcagg
gcggcagggtgcagtggtggtggagtaggcagcagctgtggtggttgtgggaggggaaatggtggctaggcgggcaagatgggctacccggtgcaggtgcgggcaggggtgtgccg
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gcgcaatcaagcgcgccgcacgcagctggcttgtatggctgtgcgtgcctcgggctctaaggcgtgcatttttcgctcggtcggagctgccagtactactgcgttgctcgatgacccctctg
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ACAATGTGCAGGAGGCCAACCGCGAGGCCGAGGGCTTCATGATCCGCGACGGCATCGTGTGCGTCATCAAGGACAGCGTCATCCCCAGCGGCACCGT
CATCTGA

Figure 5.A2 DNA sequence of the ADP-Glucose pyrophosphorylase small subunit gene of *S. obliquus*. The DNA sequence shows non-coding regions (introns) in yellow and lowercase, coding regions (exons) are shown in uppercase. The start, STOP, and mutation site are indicated in red.

Chapter 6

Transformation optimization method for microalgae

To be submitted as:
Transformation optimization method for microalgae
Lenny de Jaeger, Ka Y.F. Lip, Justus Olijslager, Jan Springer, Emil J.H. Wolbert, Dirk E. Martens, Mark H.J. Sturme, Gerrit Eggink, René H. Wijffels

The development of transformation protocols for new and less well studied microalgae is challenging. The unexplored physiology and largely unknown genomes are a bottleneck in the successful transformation of these species. This leads to the use of suboptimal genetic constructs, based on standardized vectors for plant and microalgal model species. However, one of the main reasons for unsuccessful transformation of microalgae, is poor intracellular delivery of exogenous DNA. In this study a simple and effective optimization tool for transforming less well studied microalgal species is proposed. Cell competence can be monitored by measuring cell permeabilization and viability separately using Sytox Green and Propidium Iodide respectively. This method can be used to optimize different parameters in electroporation protocols. Optimal voltage settings were determined for five microalgae: *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Neochloris oleoabundans*, *Scenedesmus obliquus*, and *Nannochloropsis sp.* The optimal voltage settings obtained for the model strains *C. reinhardtii* and *C. vulgaris* are in correspondence with the values found in literature. This method can be used to speed up the screening process for species that are susceptible for transformation and to increase the insight in established transformation protocols.

6.1 Introduction

Microalgae are a promising platform not only for the production of commercially interesting products such as oils, nutraceuticals, proteins for food and feed applications, and commodity chemicals (Chisti, 2007; Christaki et al., 2011; Draaisma et al., 2013; Gimpel et al., 2013; Pulz and Gross, 2004; Wijffels and Barbosa, 2010; Wijffels et al., 2013), but also for health beneficial products such as antioxidants (β-carotene, astaxanthin) and polyunsaturated fatty acids (Christaki et al., 2011). Genetic modification and metabolic engineering are valuable tools to speed up the development of microalgae derived products and to decrease the high production costs that are currently involved. Furthermore, transgenic microalgae could be used as production vehicles for recombinant proteins such as industrial enzymes and therapeutic proteins (Gong et al., 2011; Mayfield et al., 2007; Rasala et al., 2012). Wijffels and Barbosa (Wijffels and Barbosa, 2010) described the physiological and cellular characteristics of an "ideal microalgal cell factory" and even though extensive screening studies of microalgal isolates have been done, it is very unlikely that there will be a single species that meets all the production characteristics, such as high growth rate, high lipid productivity, product excretion, and robustness. Promising species therefore need to be further optimized, for which a generic genetic engineering toolbox is needed that can be applied to all microalgal species.

To enable the genetic modification of microalgae, an efficient transformation platform is needed. Some of the hurdles that need to be overcome are increasing nuclear transgene expression and targeting different cellular compartments in microalgae. Lately there have been good examples of tools that seem to fulfill these requirements (Lauersen et al., 2015; Rasala et al., 2014; Rasala et al., 2012). All of these advances are currently restricted to the model species *Chlamydomonas reinhardtii*, which has been the work horse for many decades in photosynthesis and metabolic engineering research and is very well characterized (Merchant et al., 2007). Although considered as a good model platform, *C. reinhardtii* is not very useful for producing triacylglycerol lipids and other fatty acid derived molecules. Some of the oleaginous microalgae that are currently being studied, and have a great potential to become part of bio-based production platforms, have not yet benefited from the genetic engineering revolution. Oleaginous microalgae that have been transformed by genetic engineering approaches include *Phaeodactylum tricornutum*, *Chlorella vulgaris*, and *Nannochloropsis sp.* (Apt et al., 1996; Chow and Tung, 1999; Kilian et al., 2011; Radakovits et al., 2012), but the available genetic toolbox for these microalgae is still in its infancy. Other industrially relevant (oleaginous) species, such as *Neochloris oleoabundans* and *Scenedesmus obliquus*, which can accumulate up to 55% of their cell dry weight as lipids (Breuer et al., 2014; Breuer et al., 2012; de Jaeger et al., 2014) and have a high lipid productivity, cannot be genetically engineered yet.

The most widely used method to deliver heterologous DNA into the production host, the first essential step in a genetic modification protocol, is electroporation (Chow and Tung, 1999; Kilian et al., 2011; Shimogawara et al., 1998). This physical method is based on generating micro-pores in the cell membrane of cells to enable the passage of exogenous plasmid DNA into the cell. This passage can be obstructed by the polysaccharide rich cell wall or even resistant extracellular terpenes such as algaenan, and the shape and size of the cell also influences successful transformation. Other physical methods include biolistics, and glass bead and carbon whisker mediated methods, which are all based on mechanical forces to get DNA inside the cell (Apt et al., 1996; Dunahay, 1993; Kindle, 1998). Finally there is the biological delivery platform using *Agrobacterium tumefaciens* (Kumar et al., 2004). In this study we describe a method for a quick and simple screening method to find optimal transformation conditions for yet untransformed microalgal species. We describe a simple and effective optimization tool using two fluorescent dyes to assess the permeability (Sytox Green) and viability (Propidium Iodide). We tested the method using electroporation on five microalgae with different physiological and cellular characteristics: *C. reinhardtii*, *C. vulgaris*, *N. oleoabundans*, *S. obliquus*, and *Nannochloropsis sp*.

6.2 Materials and Methods

6.2.1 Strains, medium and pre-culture conditions

Chlamydomonas reinhardtii (CC1690, Chlamydomonas research center), *Scenedesmus obliquus* (UTEX 393, University of Texas), *Chlorella vulgaris* (UTEX 259, University of Texas), *Neochloris oleoabundans* (UTEX 1185, University of Texas) and *Nannochloropsis sp.* (CCAP 211/78) were used in this study. *C. reinhardtii* was maintained on Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine, 1965), and all other strains were maintained in fresh water medium as described by Breuer et al. and *Nannochloropsis sp.* medium was enriched with additional salts (Breuer et al., 2012). Agar plates were made by supplementing 15 g/L agar (Duchefa Biochemie B.V., Haarlem, The Netherlands). Cultures were kept in exponential growth phase under day:night cycle regime (16:8) at 25°C at around 60 µmol m^{-2} s^{-1} light intensity (Grolux fluorescent tubes, Sylvania F36W/GRO) at 125 rpm and 2.5% CO_2.

6.2.2 Fluorescent dye delivery

Microalgal cells in the exponential phase ($5x10^6$-$1x10^7$ cells ml^{-1}) were collected by centrifugation, washed with electroporation buffer (5.0 mM KCl, 5.0 mM $CaCl_2$, 0.01 M HEPES, 0.1 M mannitol and 0.1 M sorbitol), and resuspended to a final concentration of 10^8 cells ml^{-1} in cold electroporation buffer. From this cell suspension 250 µL was transferred to a 2 mm electroporation cuvette and either Cascade Blue hydrazide (Invitrogen, Carlsbad, USA) or Sytox Green (Life Technologies, Grand Island, NY, USA; S7020) was added to a final concentration of 400 µM and 1 µM respectively, and incubated in the dark for 5

minutes on ice. These dyes were used to assess the level of permeability as a result of the electric pulse. Electroporation was carried out using a Bio-Rad Gene-Pulser apparatus set at 25µF and infinite resistance and varying field strengths (0-10.5 kV cm^{-1}). The cell suspension was recovered in the dark for 5 minutes on ice before 1 ml of electroporation buffer was added and the cells were incubated for 10 more minutes in the dark at room temperature. Cells that were assessed for cell viability were not exposed to Sytox Green or Cascade Blue but were supplemented with Propidium Iodide (Life Technologies, Inc., Carlsbad, CA) at a final concentration of 60 µM, after the recovery phase, before analysis by fluorescent microscope or flow cytometry.

6.2.3 Fluorescence screening

Cells were visually inspected with an Olympus IX71S8F-3 fluorescence microscope with 330-385 nm excitation, 420 nm emission filter and 400 nm dichromatic mirror for Cascade Blue and 460-495 nm excitation filter, 510-550 nm Emission filter and a 505 nm dichromatic mirror for Sytox Green. High throughput quantification of the dye uptake was done by using a BD Accuri C6 flow cytometry (BD Biosciences, San Jose, CA, USA). The electroporated cells were diluted 200X in electroporation buffer, before analysis on the flow cytometer to obtain optimal flow rate for detectors. Per condition 20.000 cells were measured with an argon-ion excitation laser (488 nm) and detected using three different fluorescent channels: green (FL1 530 ± 15 nm) for Sytox Green, orange (FL2 585 ± 20 nm) for Propidium Iodide, and red (FL3 670 ± 25 nm) for chlorophyll autofluorescence using a flowrate of 14 µL min^{-1}. BD Accuri C6 Software 264.21 was used for data acquisition and analysis. Forward scatter and side scatter plots were used to remove background noise and cellular debris. Sytox green uptake was analyzed by plotting log autofluorescence (FL3) versus log green fluorescence (FL1), to discriminate between cells that did and did not take up Sytox Green. Propidium Iodide uptake was analyzed by plotting log autofluorescence (FL3) versus log orange fluorescence (FL2).

6.3 Results and discussion

Transformation platforms for *C. reinhardtii* are well established and a wide range of techniques are used to metabolically engineer this model species. For promising oleaginous microalgae such as *S. obliquus* and *N. oleoabundans* genetic engineering is however hindered due to the absence of (efficient) transformation methods. There can be multiple reasons for ineffective transformation in microalgae. The first reason could be the plasmid DNA delivery in the cell. Another issue could be the transformation constructs that are used, since they are designed for plants or *C. reinhardtii* and not always functional in other microalgae, which might be caused by non-functional promoter or terminator sequences or suboptimal codon usage of the heterologous genes used. Since development of transformation protocols for microalgae can be complicated and time-consum-

ing, we therefore set out to test the application of a fluorescent dye based method to assess optimal transformation settings.

To test the first bottleneck, DNA delivery using electroporation, we developed a screening method to test the degree of permeabilization of the cell membrane after electroporation and the influence of the electric pulse on viability. To test the permeability of the cells we tested two different dyes: Cascade Blue (CB), a fluorescent tracer molecule that is non-permeable to intact cells, and Sytox Green (SG). The viability was tested using Propidium Iodide (PI). Both fluorophores SG and PI are not permeant to live cells and intercalate between the bases of nuclear DNA with little sequence preference. Intercalation of SG into double stranded DNA intensifies the fluorescence by more than 500-fold (SG), and 20-30 fold in the case of PI. This is an advantage, since the treated cells do not need to be washed before further analysis, as is the case for the non-DNA binding dye CB which always shows the same fluorescence intensity.

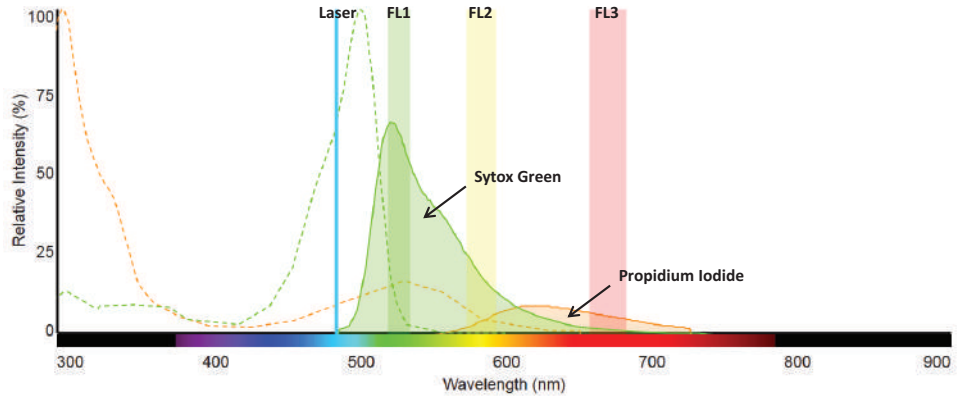

Figure 6.1 Spectrum for fluorophores and available filters. Dotted lines represent excitation spectra and solid line is the emission spectrum of Sytox Green (Green) and Propidium Iodide (Brown). Filters are indicated with their respective band width. The emission spectra are normalized to the amount of light absorbed at 488 nm.

Initially we aimed to develop a one-step screening method that would show the level of permeability and viability of the tested settings simultaneously, but the emission spectra of both intercalating fluorophores overlapped on the flow cytometer analysis, and the fluorescence level of SG was much higher than PI. Although, FL3 would show the PI fluorescence and less of the SG, the autofluorescence of the chlorophyll would obscure the measurement (Figure 6.1). The excitation and emission maxima of SG and PI are 504 nm and 523 nm and 535 nm and 617 nm respectively. For these reasons we decided to use a two-step approach in which we would test the permeability and viability effects after electroporation separately. We could have used one single dye for both the permeability

and viability screening, but for the sake of clarity we used two different fluorophores. CB can only be measured on a flow cytometer equipped with an UV lamp, and therefore SG (FL1) and PI (FL2) are used as fluorophores in the proposed method.

Our first target microalga for the dye-based method was *S. obliquus* UTEX 393, which has a highly resistant algaenan-containing cell wall. Guo et al. had described the successful transformation of the related *S. obliquus* strain FSP-3 (Guo et al., 2013), but our attempts to transform *S. obliquus* UTEX 393 using their electroporation protocol with different voltage settings did not yield in successful transformants. *Agrobacterium tumefaciens* mediated transformation and biolistics were also tested, without success. Using the microalgae *S. obliquus* we developed a microscope based protocol to test uptake of molecules after an electric pulse was applied. Cells were electroporated and recovered in the presence of CB and washed to remove excess CB. The cells were observed visually under a fluorescent microscope using a 288 nm UV lamp. Cells that created micro-pores upon electroporation were able to take up the dye and would show bright blue fluorescence.

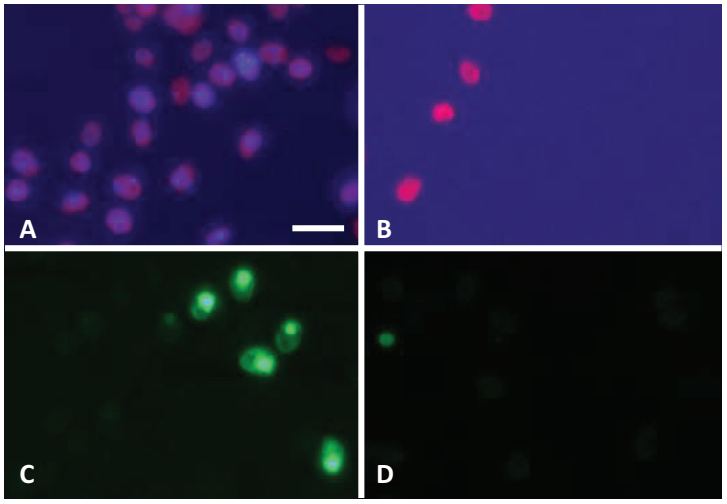

Figure 6.2 Microscopic images after electroporation (1.5 kV cm-1) of *C. reinhardtii* (A and C) and *S. obliquus* (B and D) using two different fluorophores: Cascade Blue (A and B) and Sytox Green (C and D) in. The white bar represents 10 µm.

Both *C. reinhardtii* and *S. obliquus* cells were tested at different voltage settings. In figure 6.2A-B the pictures are shown of the uptake of CB in *C. reinhardtii* and *S. obliquus* after electroporation. It is clear that *C. reinhardtii* takes up the dye when standard electroporation settings (1.5 kV cm^{-1}) are applied. *S. obliquus* does not show any uptake of the CB tracer dye under the tested conditions. We concluded that the DNA delivery in *S. obliquus* is indeed an obstacle that needs to be overcome in order to develop a successful transformation protocol using electroporation.

Although the CB tracer dye gives insight into the permeability of cells upon electroporation, there are some disadvantages to the method. This dye cannot be used in a high-throughput flow cytometer platform, since the dye requires a UV lamp to generate the fluorescent signal. The flow cytometer used in this study is not fitted with such an UV lamp. Another disadvantage is that the cells need to be washed after the tracer dye is applied, since the dye is fluorescent by itself, and without washing the remaining dye away, will obscure the measurement. In figure 6.2A-B the purple background fluorescence can be clearly seen, although *S. obliquus* cells turn pink, they do not show the purple granular spots which are visible in *C. reinhardtii*, indicative of nonspecific staining of the cell walls without actual dye uptake. Cells that have been electroporated are generally very delicate and shear sensitive, so washing by centrifugation is not desired. Finally this method does not give any information on the viability of the cells. Electroporation is based on generating micro-pores in the cell membrane of cells to enable the passage of foreign DNA into the cell. Higher voltages applied would likely result in larger pores and a higher uptake efficiency of the tracer dye, but subsequently also to a higher death rate due to the high electric pulse.

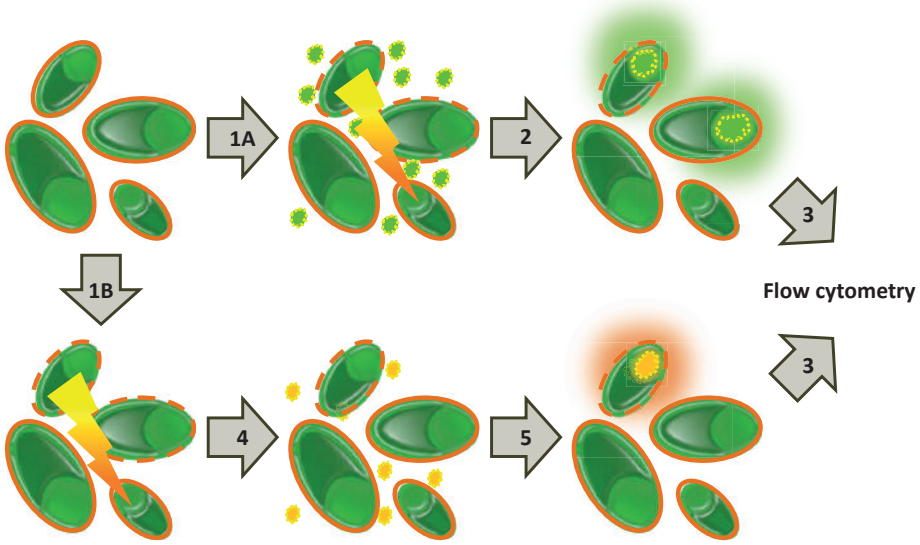

Figure 6.3 Experimental design for cell competence screening. (1A) Electroporation in the presence of SG. (2) Recovery phase. (3) Fluorescence uptake monitored with flow cytometer. (1B) Electroporation without fluorescent dye. (4) At the end of the recovery PI is added. (5) PI enters nonviable cells. Orange line represents the cell membrane and cell wall.

Electroporated cells could be observed under the microscope to study in a qualitative manner the fluorescence of CB, but not quantitative. Therefore, another experiment was designed to use fluorescent dyes that cannot pass intact cell membranes and emit a high fluorescence signal upon binding to DNA, and can be analyzed in a flow cytometer.

In figure 6.2C-D fluorescent microscopic pictures are shown of *C. reinhardtii* and *S. obliquus* cells electroporated in the presence of SG. The same settings were applied compared to the CB experiment. It can be seen that the background noise level is much lower and the fluorescent signal upon uptake of the fluorophore much higher compared to CB. SG was not able to enter *S. obliquus* cells upon electroporation as seen with CB before. *C. reinhardtii* however was able to take up the fluorescent dye in the tested conditions (Figure 6.2). Based on the information on dye specificities and microscopic observations on dye uptake we tested five microalgal species (with different characteristics) using the experimental design described in figure 6.3. Cells were electroporated in the presence of SG and, after recovery, measured by flow cytometry to determine the permeable population. A second portion of the cells was electroporated and had PI added after the recovery phase, to determine the cell population that did not close the pores that were induced by the electric pulse. These cells are considered to be non-viable.

In figure 6.4 the viability and permeability for five different microalgae is shown for different voltage settings (For examples of flow cytometer output see Appendix Figure 6.A1). All species show a similar pattern. The higher the voltage that was applied the higher the SG uptake was, representing the permeabilized population. The viability decreases when a higher voltage was applied. Viability measured by PI uptake were supported by colony forming unit (CFU) values obtained by plating of electroporated cells. It seems that there is a tipping point around which the viability decreases and the permeability increases rapidly. The voltage that corresponds to the moment that the two lines intersect is not the same for all species.

From the tested species, *C. reinhardtii* is the most prone to taking up SG and the intersect of the viability and permeability curves lies around 2.25 kV cm^{-1}. *C. vulgaris* has the tipping point slightly higher than *C. reinhardtii* around 5 kV cm^{-1}. The oleaginous microalgae *N. oleoabundans* and *S. obliquus* seem to be even more resistant and have the tipping point around 6 and 7 kV cm^{-1} respectively. The marine species *Nannochloropsis sp.* shows a different curve and the viability is only 68% even without electroporation, meaning that 32% of the cells have taken up SG and are nonviable before the pulse is applied. This could be explained by the fact that we use a low salt electroporation buffer to enable high voltage settings, which causes an osmotic shock that probably kills a large fraction of the cells. If the salinity of the buffer would be increased, the osmotic shock and probably the high death rate would be reduced, but arcing at higher voltages was observed and therefore the use of low salt buffers was required. The electroporation buffers that are described in literature for *Nannochloropsis sp.* are similar to the one we tested here (Radakovits et al., 2012). Due to the high mortality rate there is no tipping point to be observed for *Nannochloropsis sp.*

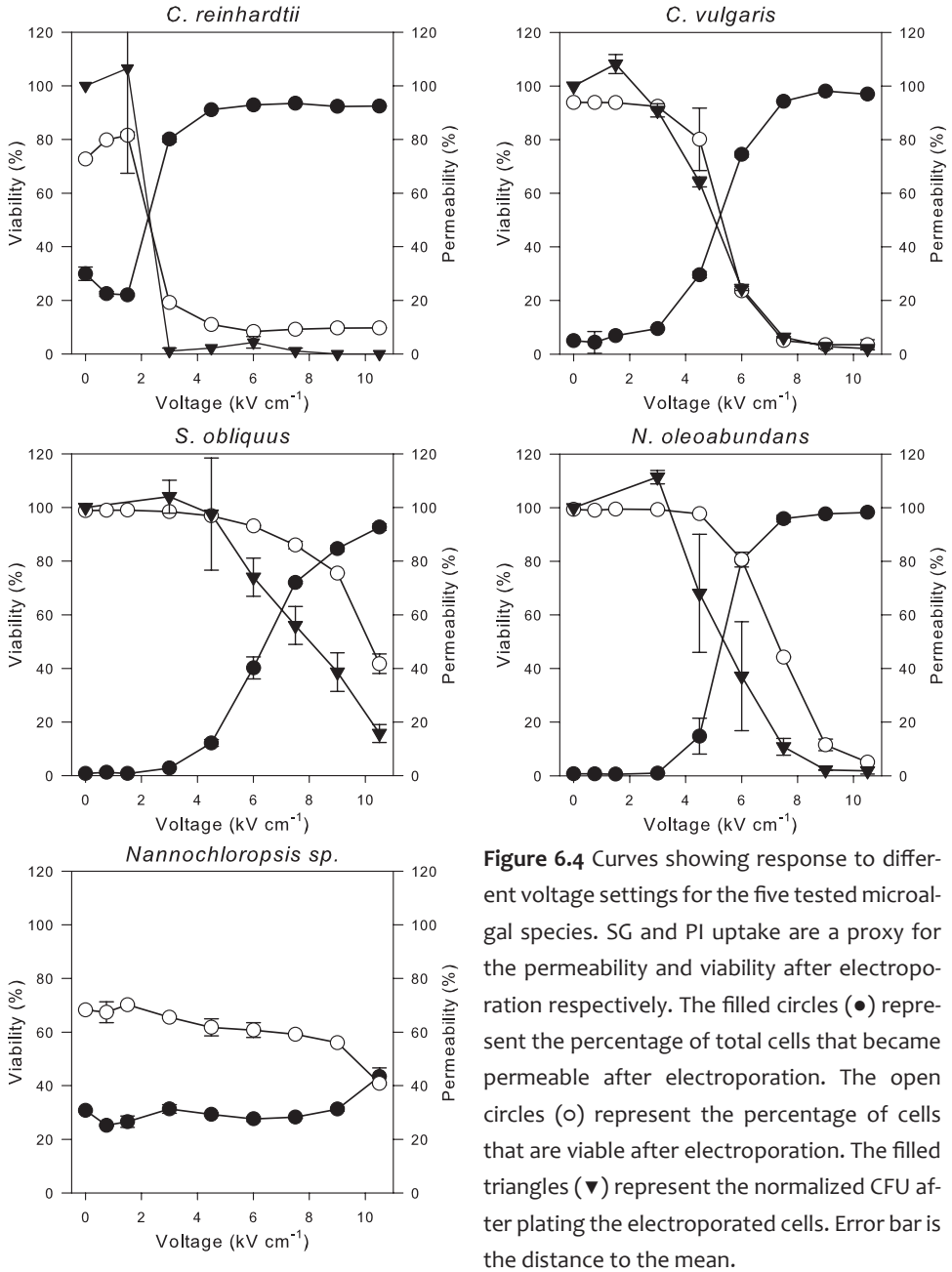

Figure 6.4 Curves showing response to different voltage settings for the five tested microalgal species. SG and PI uptake are a proxy for the permeability and viability after electroporation respectively. The filled circles (●) represent the percentage of total cells that became permeable after electroporation. The open circles (○) represent the percentage of cells that are viable after electroporation. The filled triangles (▼) represent the normalized CFU after plating the electroporated cells. Error bar is the distance to the mean.

The difference in response of permeability and viability differs from species to species. We hypothesize that this is caused by the structure and composition of the cell wall. It is known that species such as *S. obliquus* and *Nannochloropsis sp.* have a more rigid cell wall than *C. reinhardtii*. The cell wall of *C. reinhardtii* lacks cellulose and is composed

of hydroxyproline-rich glycoproteins (Macfie et al., 1994; Voigt, 1988). *S. obliquus* has a more complex cell wall with several layers. The inner layer consists of cellulose and around this cellulose layer is a trilaminar structure which is known as algaenan. The exact design of the trilaminar algaenan structure is still unknown, but it has been characterized by its physical and chemical properties (Allard et al., 1998). Layers 1 and 3 of the trilaminar structure are resistant against acetolysis and enzymatic degradation by known enzymes and it is suggested that these layers contain glycoproteins and glucosamine containing biopolymers (Burczyk et al., 1999). The middle layer is osmophobic which suggests the presence of lipids (Burczyk et al., 1970). The cell wall of *Nannochloropsis gaditana* (strain CCMP 526) has a similar organization to *S. obliquus*, and has a cellulosic inner wall protected by an outer hydrophobic algaenan layer (Scholz et al., 2014). There is not much reported on the cell wall composition of *Nannochloropsis sp.* in literature. *C. vulgaris* has a cell wall containing glucosamine, cellulose and other polysaccharides containing mainly rhamnose and arabinose (Pieper et al., 2012). The cell wall of *N. oleoabundans* has not been studied in much detail, and contains cellulose (Chantanachat and Bold, 1962; Wang et al., 2015)1962; Wang et al., 2015, yet there are no reports describing the presence of an algaenan cell wall. Based on the outline of the cell wall of the tested species we would expect that *C. reinhardtii* and *C. vulgaris* have the most accessible cell wall followed by *N. oleoabundans*, and *S. obliquus* and *Nannochloropsis sp.* that have the most rigid cell wall. This corresponds to the outcome of the permeability test as can be seen in figure 6.4.

Almost all species show an increase in viability in the CFU count after electroporation in the low range 0.75-3 kV cm^{-1}. This could be the result from dissociation of small aggregates of microalgal cells that resulted from the washing steps prior to the electroporation. The flow cytometer will find more small events when the cells are dissociated by electroporation and larger aggregates when no electric pulse is applied. The delicate balance between viability and permeability makes electroporation a platform that needs optimization. To extract the optimal electroporation settings for the tested species we suggest to calculate the competent cell fraction by using the following equation:

$$Competent\ Cell\ Fraction = Population\ fraction_{SG\ uptake} - Population\ fraction_{PI\ uptake} \quad \text{(Eq. 6.1)}$$

Competent cells are defined as the cells that form micro-pores upon electroporation, are able to close these pores and are viable thereafter. Note that using this equation it is assumed that all intact cells are viable, but there might be some nonviable cells that do not take up PI, so there could be a slight overestimation of the viable cell population. In figure 6.5 the competent cell fraction is calculated for all strains for every voltage that was tested. All fresh water species show a similar pattern for the competent cell fraction and an optimal voltage setting can be assigned. The salt water species *Nannochloropsis*

sp. shows a different curve where no clear optimal voltage setting can be assigned. Two possible reasons are the low viability due to the osmotic shock in the electroporation buffer and the voltage settings that are lower when compared to literature. The latter can be explained by the choice for a generic protocol, therefore all settings were standardized except the voltage. Higher voltages can be obtained when 1 mm electroporation cuvettes are used instead of the 2 mm cuvettes in this study. In table 6.1 the optimal voltage settings for every species is shown and compared to electroporation settings described in literature.

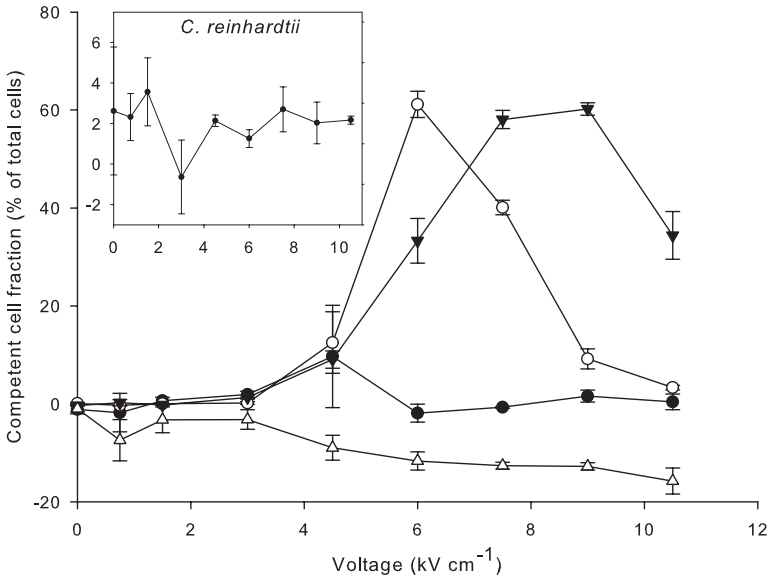

Figure 6.5 Competent cell fraction for all tested species. The values show the fraction of viable cells that have been permeable for each voltage setting tested. C. reinhardtii is shown in a separate window. C. vulgaris (●), N. oleoabundans (○), S. obliquus (▼), and Nannochloropsis sp. (Δ). Error bars show the distance to the mean.

The values that are obtained in this study are slightly lower than the values described in literature, but in general correspond quite well. This is a good indication for the validity of the presented method. The fluorophores used in this study are smaller than the plasmid DNA that is normally used. Thus the method might give a wrong interpretation of transformability, since the pore size is less restrictive for the smaller fluorophores than the (linearized) plasmid DNA. To further improve the method it would therefore be good to repeat these studies with fluorescently labeled plasmid DNA instead of fluorescent dyes. Finally the optimal settings should be put to practice to test the DNA transformation efficiency for each setting to see if the predicted values correspond to the maximum efficiency obtained.

Table 6.1 Optimal voltage settings for the tested microalgae in this study compared to optimal voltage settings described in literature.

Microalgal species	Proposed voltage (kV cm-1)	Voltage from literature (kV cm-1)	Reference
C. reinhardtii	1.5	2	(Rasala et al., 2012)
C. vulgaris	4.5	5	(Koo et al., 2013)
N. oleoabundans	6	n.d.	-
S. obliquus	9	10*	(Guo et al., 2013)*
Nannochloropsis sp.	n.d.	11-12*	(Radakovits et al., 2012)*

n.d. Not Determined (outside tested range)

*Not the same species as tested in this study

6.4 Conclusions

A simple and effective electroporation optimization tool for microalgal species is proposed. Cell competence can be monitored by measuring cell permeabilization and viability separately using Sytox Green and Propidium Iodide respectively. This method can be used to optimize parameters in electroporation protocols, such as voltage, resistance, cuvette size and osmotic buffer. But also optimization of other transformation tools such as glass bead and Polyethylene Glycol mediated transformation could benefit from this method. The obtained optimal voltage settings are in correspondence with the values found in literature for DNA transformation of the model species *C. reinhardtii* and *C. vulgaris* and the oleaginous species *S. obliquus* (strain FSP-3). This demonstrates that the method is a good indicator whether conditions tested are suitable for actual DNA transformation. Using this method, foreign DNA delivery in the target species can now be studied and thereby the bottleneck of DNA delivery can be ruled out when transformation remains unsuccessful.

Acknowledgements

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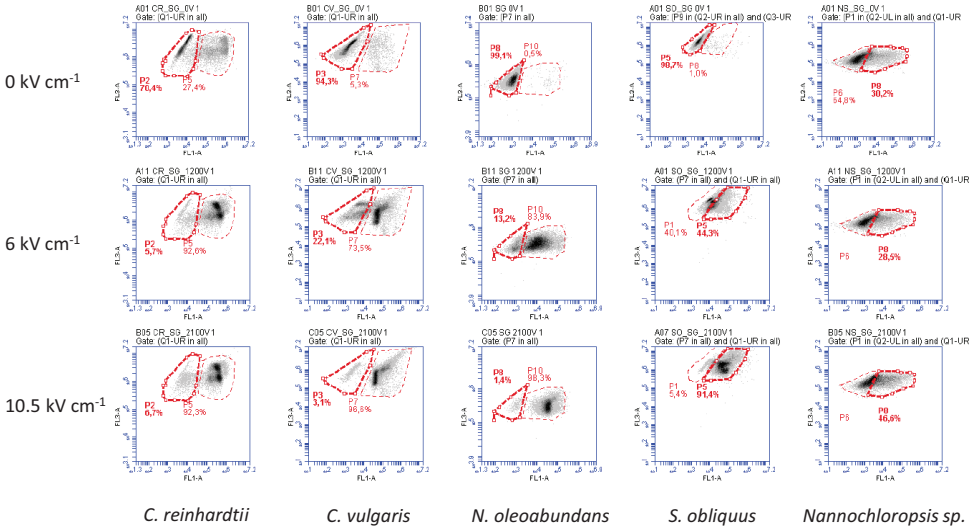
Appendix

Permeability (Sytox Green)



Viability (Propidium Iodide)

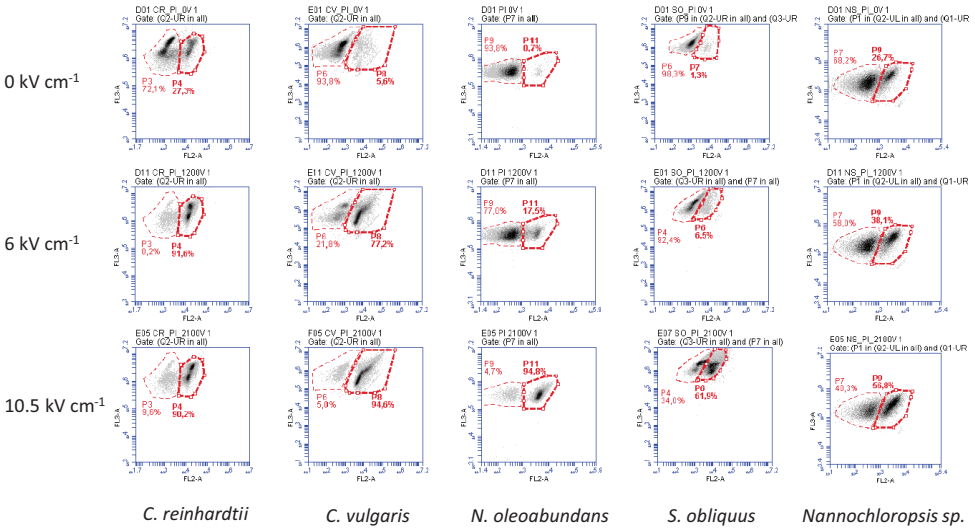


Figure 6.A1 Flow cytometer output

Chapter 7

Gene silencing of stearoyl-ACP desaturase enhances the stearic acid content in *Chlamydomonas reinhardtii*

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Gene silencing of stearoyl-ACP desaturase enhances the stearic acid content in Chlamydomonas reinhardtii
Lenny de Jaeger, Michiel Herpers, Jan Springer, Emil J.H. Wolbert, Dirk E. Martens, Gerrit Eggink and René H. Wijffels

The current increase in oil consumption, combined with predicted population growth for the coming decades, requires an alternative, sustainable and renewable source of oils. Microalgae that accumulate the saturated fatty acid stearic acid are of special interest as an alternative to existing vegetable sources. In this study, stearoyl-ACP desaturase (SAD), the enzyme that converts stearic acid into oleic acid, is silenced by artificial microRNA in the green microalga *Chlamydomonas reinhardtii*. Two different constructs, which target different positions on the mRNA of stearoyl-ACP desaturase, were tested. For each construct, three transformants were cultivated and the total fatty acid and triacylglycerol content was measured at two temperatures over time. The mRNA levels for SAD were reduced after the silencing construct was induced. In one of the strains, the reduction in SAD mRNA resulted in a doubling of the stearic acid content in triacylglycerol molecules, which shows that stearic acid production in microalgae is possible. Furthermore, we hypothesize that in addition to direct conversion in the chloroplast, *C. reinhardtii* is able to redirect stearic acid from the chloroplast to the cytosol and convert it to oleic acid in the endoplasmic reticulum by stearoyl-CoA desaturase.

7.1 Introduction

The current increase in oil consumption, combined with predicted population growth for the coming decades, requires an alternative, sustainable and renewable source of oils. Most renewable oil resources are based on extraction of oil from plant species such as palm oil, rapeseed, corn and others (Carlsson, 2009). Increased use of these resources will have a great impact on the available arable land, fresh water, and delicate nature. An alternative oil production crop are microalgae, which have a very high areal productivity compared to land plants (Chisti, 2007).

Currently, the most used feedstock for vegetable oil is derived from palm, in which palmitic acid accounts for 45% of the total oil in the seeds (Table 7.1). Although palmitic acid rich oils are suitable for high temperature applications (Guinda et al., 2003), it is known that saturated fatty acids, can raise the health risk by increasing low-density lipoprotein cholesterol (Cox et al., 1995). Furthermore saturated fatty acids are regarded as unhealthy as high intake is associated cardio-vascular diseases (Mensink et al., 2003). Structural properties of oils are a result of the higher melting point of these saturated fatty acids compared to the lower melting point of mono- and polyunsaturated fatty acids present in triacylglycerol. Oils rich in saturated fatty acids are semi-solid at room temperature and can therefore be directly used in products that require structural properties (Fernandez-Moya et al., 2002).

Oils that are low in palmitic acid and rich in stearic acid and oleic acid can provide the structural properties and do not have the negative health effect that is attributed to palmitic acid and therefore the presence of stearic acid rich oils in the diet is preferred over palmitic acid rich oils (Mensink et al., 2003).

An effective chemical method to produce saturated fatty acids is, hydrogenation of mono- and polyunsaturated fatty acids removing the double bonds. Disadvantage of this method is, that partial hydrogenation will lead to trans fatty acids, which are known to have potentially negative health effects and can raise cholesterol levels and therefore this process is not desirable (Ascherio and Willett, 1997).

There are only few crops known that naturally produce large amounts of stearic acid, for example Shea and Allanblackia (Table 7.1). But oils derived from those sources are expensive compared to the palmitic rich oils derived from palm oil and hydrogenated oils. There are various examples of organisms that have an enhanced stearic acid content derived via selective breeding, mutagenesis and targeted genetic engineering. Using hairpin mediated RNA silencing Liu et al. generated cottonseeds with elevated stearic acid contents up to almost 40% of total fatty acids (Liu et al., 2002). Other crops with

enhanced stearic acid contents are soybean (chemical mutagenesis) (Graef et al., 1985), canola (antisense) (Knutzon et al., 1992), *Arabidopsis thaliana* (chemical mutagenesis) (Lightner et al., 1994), and also the yeast *Apiotrichum curvatum* (chemical mutagenesis) (Ykema et al., 1989).

Table 7.1 Fatty acid composition of several oil rich plants and microalgae.

Species	Fatty Acid (CDW%)							Reference
	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	Other	
Higher Plants[a]								
Elaeis guineensis (Palm)	45	5	39	11	0	0	1	(Nzikou et al., 2010)
Butyrospermum parkii (Shea)	4	42	46	7	0	1	0	(Maranz et al., 2004)
Theobroma cacao (Cacao)	25	35	35	3	0	0	2	(Ramadan et al., 2006)
Allanblackia floribunda	1	56	43	0	0	0	0	(Foma et al., 1985)
Gossypium hirsutum (Cotton)	26	2	13	59	0	0	0	(Liu et al., 2002)
Glycine max (Soybean)	16	3	13	58	10	0	0	(Singh et al., 2010)
Microalgae[b]								
Chlamydomonas reinhardtii	29	4	12	12	28	2	15	(James et al., 2011)
C. reinhardtii BAFJ5	30	3	24	16	16	1	10	(James et al., 2011)
Chlorella vulgaris	17	2	47	10	14	0	10	(Breuer et al., 2012)
Chlorella zofingiensis	15	3	47	17	8	0	10	(Breuer et al., 2012)
Dunaliella tertiolecta	25	2	14	9	31	0	19	(Breuer et al., 2012)
Neochloris oleoabundans	23	3	41	21	3	0	9	(Breuer et al., 2012)
Porphyridium cruentum	28	4	5	30	0	0	33	(Breuer et al., 2012)
Phaeodactylum tricornutum	27	1	3	1	0	0	68	(Breuer et al., 2012)
Scenedesmus obliquus	14	5	50	7	8	0	16	(Breuer et al., 2012)

[a]The values given for higher plants are fatty acid compositions of the oil rich seeds.

[b]These values are representing fatty acid profiles for nitrogen starved cells to induce lipid accumulation.

Microalgae have an enormous potential for producing stearic acid, because the TAG molecules, which are produced under unfavorable growth conditions in several strains, contain oleic acid as the predominant fatty acids (Table 7.1). The green microalgae *Chlamydomonas reinhardtii* has been used for many years as a model organism in research, which makes it the most extensively studied green microalgae available. *C. reinhardtii* has a very well-studied physiology, a sequenced and annotated genome, and there are many tools available that enable genetic and metabolic engineering (Day and Goldschmidt-Clermont, 2011; Merchant et al., 2007; Molnar et al., 2009).

In this study *C. reinhardtii* was used to show the possibility of increasing the stearic acid content in algae by means of genetic modification. Wild type *C. reinhardtii* does not accumulate large amounts of TAG under unfavorable growth conditions and therefore the starchless mutant BAFJ5 was selected. The BAFJ5 starchless mutant is derived from *C. reinhardtii* and has a disrupted small subunit of ADP-glucose pyrophosphorylase (Zabawinski et al., 2001), and is unable to synthesize starch and therefore can accumulate more TAG than the wild type strain. The oleic acid content is elevated in the BAFJ5 starchless mutant under nitrogen depleted conditions due to the oleic acid rich TAG molecules that are accumulated under these conditions (James et al., 2011) (Table 7.1). The soluble stearoyl-ACP desaturase (SAD) enzyme introduces a cis-double bond in stearic acid, resulting in the mono-unsaturated fatty acid oleic acid. Reducing the activity of this enzyme could potentially result in an enrichment of stearic acid in the TAG molecules. The effect of reducing the expression of the (SAD) gene using artificial microRNA (amiRNA) was studied. RNA interference (RNAi) is a tool to knockdown gene expression at the post-transcriptional level and in that way enables the study of gene function and the reprogramming of specific metabolic processes.

7.2 Material and methods

7.2.1 Strains, growth conditions

The starchless mutant BAFJ5 (cw15 sta6), derived from *Chlamydomonas reinhardtii* 330 (mt1 arg7-7 cw15 nit1 nit2), which has a disrupted small subunit of ADP-glucose pyrophosphorylase (Zabawinski et al., 2001) was obtained from the Chlamydomonas Resource Center (University of Minnesota). All strains were grown on Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine, 1965) with 15g/L agar for plates.

7.2.2 Construction of transformation vector

For the knockdown of the plastid stearoyl-ACP desaturase (also known as the FAB2 gene, XM_001691545) gene expression, artificial microRNA (amiRNA) sequences were designed using the web tool Web Micro RNA designer 3 (http://wmd3.weigelworld.org). Two different targets on the SAD gene were chosen and specific amiRNA constructs were developed. One sequence was designed to target the end of the eighth exon of the SAD mRNA, and the other target was the second exon of the SAD mRNA. The 90 base pair long oligonucleotides that were used to make the silencing construct can be found in table 7.2. Both constructs were cloned into the pChlamiRNAi3int vector (Chlamydomonas Resource Center at University of Minnesota) according to Molnar et al. (Molnar et al., 2009) to generate pΔ9DSamiRNA_I and pΔ9DSamiRNA_II respectively. An empty pChlamiRNAi3int plasmid was used as a negative control during the whole experiment.

Table 7.2 Primers used in this study. amiRNA; artificial microRNA construct I and II. SAD; Stearoyl-ACP desaturase. CBLP; *Chlamydomonas* beta subunit-like polypeptide, reference gene. β–tub; β-tubulin, reference gene

Fwd amiRNA I	ctagtCTGCATTATGTAGCCAATTAAtctcgctgatcggcaccatgggggtggtggtgatcagcgctaTTAAATGGCTACATAATGCAGg
Rvs amiRNA I	ctagcCTGCATTATGTAGCCATTTAAtagcgctgatcaccaccacccccatggtgccgatcagcgagaTTAATTGGCTACATAATGCAGa
Fwd amiRNA II	ctagtTCGGAGTATGTTCCTTGCGTAtctcgctgatcggcaccatgggggtggtggtgatcagcgctaTACGTGAGGAACATACTCCGAg
Rvs amiRNA II	ctagcTCGGAGTATGTTCCTCACGTAtagcgctgatcaccaccacccccatggtgccgatcagcgagaTACGCAAGGAACATACTCCGAa

Name	**Fwd 5'-3'**	**Rvs 5'-3'**	**Accession**	**Source**
SAD	CCTCGTGTGCTGAATGTTGT	CTGCACCCTGTGACTTCTGA	XM_001691545.1	This study
CBLP	CTTCTCGCCCATGACCAC	CCCACCAGGTTGTTCTTCAG	ACB05909.1	(Moseley et al., 2006)
β-tub	GCCTCTTCCTCTTCGTTTCAGT	GAGACGACCTCCCAGAACTTG	XM_001693945.1	This study
Paro^R	CCCTCAGAAGAACTCGTCCAACAGC	GAGGATCTGGACGAGGAGCGGAA		This study
Insert	GGTGTTGGGTCGGTGTTTTTG	TAGCGCTGATCACCACCACCC		This study

7.2.3 Transformation

The BAFJ5 starchless mutant was grown to mid log phase (around $5x10^6$ cells ml^{-1}), concentrated to $3x10^8$ cells ml^{-1} by centrifugation and resuspended in TAP medium enriched with 40mM sucrose. From this cell suspension, 250 µl was mixed with 1 µg linearized plasmid, digested with *Xmn*I, and transferred to a 4 mm electroporation cuvette. Cuvettes were incubated at room temperature for 10 minutes, before electroporation using the Gene-Pulser (Bio-Rad) with the following settings: 600 V cm^{-1}, 25 µF and infinite resistance. Cells were allowed to initially recover for 15 min at room temperature, diluted to a total volume of 10 ml in TAP-sucrose (40mM) and allowed to recover in low light for 24 hours at 25°C. After recovery the cells were concentrated and plated on TAP selection plates, containing 10 µg ml^{-1} paromomycin (Duchefa, Haarlem, The Netherlands). Plates were incubated at 25°C for 9 days in continuous light (50 µmol m^{-2} s^{-1}). Transformants were assessed for their oleic acid dependence by replica-plating the colonies on TAP agar plates with and without 100 µM oleic acid. All plates contained 1g l^{-1} Tween 80 to disperse the oleic acid in the medium.

7.2.4 Experimental set up

Strains were cultivated in 100 ml TAP medium in 300 ml Erlenmeyer flasks in continuous light (100 µmol s^{-1} m^{-2}) at either 25°C or 35°C and shaken at 125 rpm. Each strain was grown in duplicate and inoculated 48 hours before medium replacement at an OD_{750} of approximately 0.5. After 48 hours the cells were harvested by centrifugation and washed twice with nitrogen depleted TAP medium and resuspended in 100 ml of nitrogen free TAP medium to induce the TAG accumulation. Immediately after the medium replacement (T=0h), the cells were heat shocked for 60 min at 40°C to induce transcription of the amiRNA construct. Samples were taken at -24h, 8h, 24h, 48, and 72h to measure the dry weight content, total fatty acid and triacylglycerol content, and to extract RNA for real-time quantitative PCR.

7.2.4 Determination of dry weight concentration

Dry weight concentrations were determined at -24h, 8h, 24h, 48h, and 72h on biological replicates. Around 1.5 mg of biomass was filtered through Whatman glass fiber filter paper (GF/F; Whatman International Ltd, Maidstone, UK) (de Jaeger et al., 2014).

7.2.5 Total fatty acid and triacylglycerol analysis

Total fatty acid (TFA) and triacylglycerol (TAG) extraction and quantification were performed as described by Breuer et al. (Breuer et al., 2013a) with the following adjustments. Around 10 mg of biomass was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) and lyophilized and disrupted by bead beating in the presence of a chloroform:methanol mixture (1:1.25) to extract the TFA from the biomass. Two internal standards were added to the chloroform:methanol mixture to enable quantification of both the polar lipids and TAG content. 1,2-dilauroyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt (Avanti polar lipids) and tripentadecanoin (Sigma-Aldrich, St Louis, MO, USA) were used as internal standards for polar lipid and TAG quantification respectively. The chloroform-methanol mixture was evaporated under N_2 gas and the TFA pellet was dissolved in hexane and separated based on polarity using a Sep-Pak Vac silica cartridge (6 cc, 1,000 mg; Waters, Milford, MA, USA). The neutral TAG fraction was eluted with 10 ml of hexane:diethyl ether (7:1 v/v) and the polar lipid fraction containing the glycolipids and phospholipids (PL) was eluded with methanol:acetone:hexane (2:2:1 v/v/v). Methylation of the TAG and polar lipid fraction to fatty acid methyl esters (FAMEs) and the quantification of the FAMEs were performed as described by Breuer et al (Breuer et al., 2013a). TFA concentration was calculated as the sum of the polar and neutral lipid fraction.

7.2.6 DNA extraction

Around 2 g (wet pellet) was ground for 5 min in liquid nitrogen using a mortar and pestle to disrupt the cells. DNA was isolated using hot phenol-chloroform extraction followed

by isopropanol sodium acetate precipitation. The unwanted RNA that was extracted simultaneously was removed using 10 mg μl^{-1} RNase-A treatment for 60 min at 37°C. The quality of the extracted DNA was checked by agarose gel electrophoresis and quantified using a NanoDrop 1000 spectrophotometer (NanoDrop).

7.2.7 Southern blot

Genomic DNA (2.5 µg) was digested with *Bam*HI, and transferred to positively charged nylon membrane by capillary blotting. Digoxigenin (DIG) labeled probe was generated by PCR using the PCR DIG labeling mix (Roche Diagnostics, Almere) (Table 7.2). Membranes were sealed in hybridization tubes (Techne) with DIG easy hyb solution (Roche). The pre-hybridization (2h) and hybridization (o/n) with DIG labeled probe (20 ng/ml) was performed at 42 °C. After the overnight incubation, the membrane was washed twice in low stringency buffer (2X SSC/0.1% SDS) and high stringency buffer (0.2X SSC/0.1% SDS). The probe-target hybrids were detected with an enzyme-linked immunoassay according to the DIG application manual from Roche.

7.2.8 RNA extraction

Samples for RNA extraction (2 ml) were immediately processed after sampling by 3 min centrifugation at 12.000xg at 0°C and snap frozen in liquid nitrogen before storage at -80°C until further processing. The RNA was extracted using the RNeasy Plant mini kit (Qiagen) according to the manufacturer's recommendations, with the exception, that the initial cell lysis was by bead beating the cells for 10 seconds at 5500 rpm (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) with a Precellys 24 homogenizer (Bertin Technologies, Orléans, France). After the first wash step, on column DNA digestion was performed using RNase-Free DNase kit (Qiagen). RNA was eluted in 40 µl RNAse free water and quantified and quality controlled using the NanoDrop 1000 spectrophotometer (NanoDrop) and gel electrophoresis.

7.2.9 Complementary DNA synthesis and real-time quantitative PCR

Single-stranded complementary DNA (cDNA) was obtained from 1 µg of total RNA using the reverse transcriptase system (Promega) following the suppliers protocol. The knockdown of the SAD gene was studied by real-time quantitative PCR (qPCR) using SAD specific primers (Table 7.2). cDNA was PCR amplified with Platinum Taq DNA polymerase (Invitrogen). The cDNA was analyzed by qPCR using SYBR green dye and a MyIQ thermal cycler (Bio-Rad) using the following program: 8 min at 94°C, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min. Each qPCR assay was executed in duplicate using RNA isolated from independent cultures and values were normalized using *C. reinhardtii* housekeeping genes *Chlamydomonas* beta subunit-like polypeptide (CBLP) and β-tubulin (βtub). For all samples, the starting quantity of the target gene expression was determined and compared to the geometric average of CBLP and βtub gene expression.

7.3 Results and Discussion

In this study we generated stearoyl-ACP desaturase (SAD) knockdown strains from the TAG accumulating *C. reinhardtii* starchless mutant BAFJ5 using artificial microRNA post translational gene silencing (Molnar et al., 2009). SAD, also known as delta-9 fatty acid desaturase (EC:1.14.19.2), functions by catalyzing the insertion of a double bond at the delta 9 position of stearoyl-ACP (C18:0) resulting in the monounsaturated fatty acid oleoyl-ACP (C18:1). Two different constructs were used to study the role of SAD in fatty acid synthesis in *C. reinhardtii*. The first construct (named: DSI) targeted the last exon of the SAD mRNA (Figure 7.1A) and a second construct (named: DSII) targeted the mRNA more upstream in the region that is encoded from the second exon (Figure 7.1A). A third construct was used which did not contain an amiRNA target and was used as a control vector (named: Empty). Three transformants were selected for each amiRNA construct, containing either the DSI or DSII silencing cassettes, from 40 and 35 screened transformants respectively. Two transformants were selected containing the empty vector out of 35 transformants screened. Transformants were selected based on the demon-

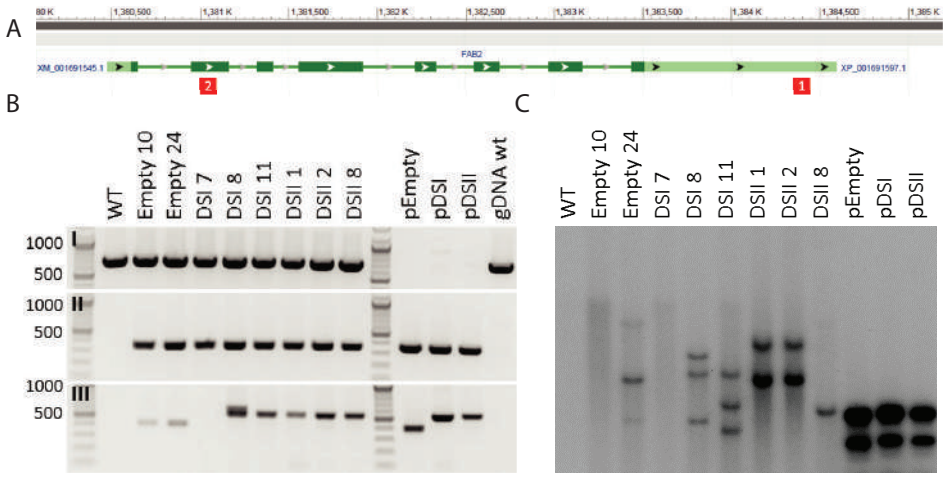

Figure 7.1 Gene map with amiRNA targets (A). Numbers 1 and 2 in red boxes correspond to DSI and DSII respectively. PCR on extracted DNA from the tested strains (B). Roman numbers correspond to 18S control (I), Paromomycin resistance gene (II), and amiRNA insert (III). Southern blot on all strains (C). A probe was used that attached to the amiRNA construct. The three linearized plasmids are included as a control.

stration of the presence of the construct by PCR (Figure 7.1B) and absence of abnormal growth characteristics. All eight transformants (empty 10, empty 24, DSI7, DSI8, DSI11, DSII1, DSII2, and DSII8) plus the wild type strain were checked by Southern blotting for the presence of the construct. Successful integration of the construct was confirmed

by Southern blotting for all the strains with the exception of strains Empty 10 and DSI7 (Figure 7.1C). All transformants tested showed a positive result for the paromomycin resistance gene in the PCR check. For the presence of the amiRNA construct all transformants were positive as well, with the exception of DSI7. An explanation could be that the silencing construct was only partially integrated in the *C. reinhardtii* genome (Figure 7.1B). The Southern blot shows that most transgenic strains contain multiple copies of the silencing construct, depicted by multiple fragments on the Southern blot.

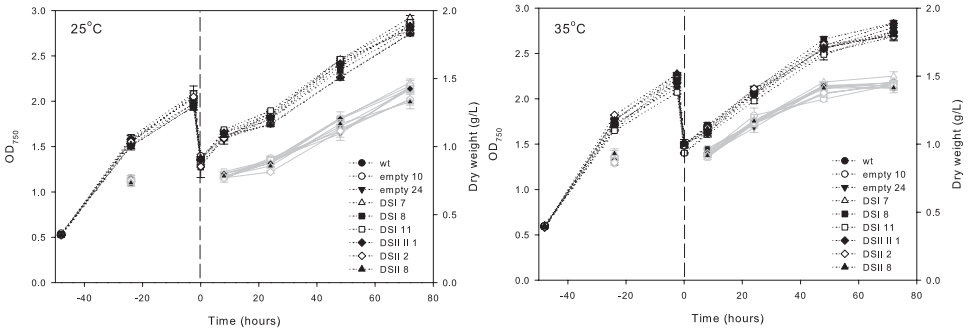

Figure 7.2 Comparison of growth rate between transgenic lines and wild type. The effect of temperature and nitrogen depletion is shown. The dotted line indicates the moment of medium replacement with nitrogen depleted TAP medium followed by a heat shock. Black dotted lines represent the OD_{750} and the gray dotted line represents the cell dry weight. Error bars indicate the distance to the mean between two biological replicates.

The transgenic lines plus the wild type strain were cultivated in shake flasks to assess the differences in growth rate and to study the effect of silencing the SAD gene on the fatty acid composition of the different strains. In Figure 7.2 the growth curves of all strains are shown, cultivated at 25°C and 35°C. All transgenic strains show a similar growth rate under nitrogen replete and depleted conditions compared to the wild type strain.

The temperature of the culture does seem to influence the growth rate of all *C. reinhardtii* strains. Cultures kept at 35°C showed a slightly higher growth rate compared to cultures kept at 25°C. The slope of the growth curves was steeper for the 35°C cultivated strains under both nitrogen replete and depleted conditions (Figure 7.2). But the growth of cultures kept at 35°C leveled off towards the end of the experiment and after 5 days the cultures died (data not shown), which has been observed before (James et al., 2013). The growth of cultures maintained at 25°C did level off after five days but no decline was observed (data not shown).

Samples were taken during the cultivation experiment to analyze the fatty acid composition of the triacylglycerol (TAG) and polar lipid (PL, phospholipids and other membrane

lipids) for the different transgenic lines. In Figure 7.3 the lipid content of all the tested strains is shown. Under nitrogen replete conditions TAGs could not be detected, since the TAG content under these conditions is too low to be measured accurately. The polar lipid fraction was always around 10% of the cell dry weight (CDW) (TFA-TAG fraction in Figure 7.3). Under nitrogen depleted conditions, there seems to be a quicker accumulation rate of TAG molecules at higher temperatures in the initial phase (8 hour) of nitrogen starvation, but the final TAG content after 72 hours of nitrogen starvation was similar for the strains cultured at both temperatures. The total amount of TAG accumulated in the transgenic lines compared to the wild type was not different. All strains accumulated around 36% of their CDW as TAG molecules and around 10% of their CDW was consisting of polar lipids during the experiment. The transgenic lines containing the DSII silencing construct have a slightly reduced TAG content, when cultured at 25°C, during the first 2 days of the nitrogen depletion phase compared to the wild type (table 7.A1, Appendix).

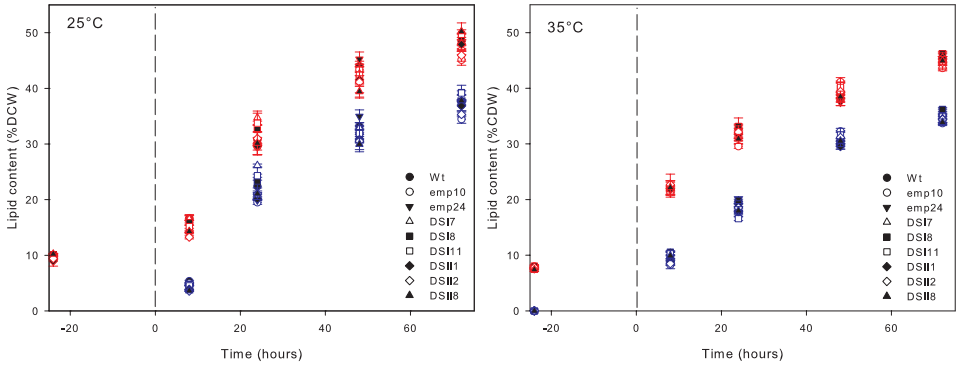

Figure 7.3 Fatty acid content. Red symbols represent the total fatty acid content and the blue symbols represent the triacylglycerol content for all tested strains. Dotted line is the time point at which the nitrogen depletion started and the heat shock applied. Error bars indicate the distance to the mean between two biological replicates.

Examination of the fatty acid profiles of the wild type and all transgenic lines, cultivated under the different temperatures conditions, demonstrates an effect of silencing the SAD gene. In table 7.3 the fatty acid profiles are shown for the first time point after nitrogen depletion showing the contribution of each measured fatty acid to the total fatty acid content. The variation in the PL between the transgenic lines and the wild type is very small. The major differences can be found in the TAG fatty acid profiles. The differences between both temperatures are noticeable mainly in the total saturated fatty acid content. At 25°C the TAG molecules in the wild type and empty vector transgenic lines contain slightly more (C14:0), and less palmitic acid (C16:0) compared to 35°C cultivated wild type strains. The stearic acid (C18:0) content is higher at elevated temperatures for wild type and empty vector containing transgenic strains (Figure 7.4 and Table 7.3), as

described for *C. reinhardtii* previously (James et al., 2013). The oleic acid content is reduced under the higher temperatures.

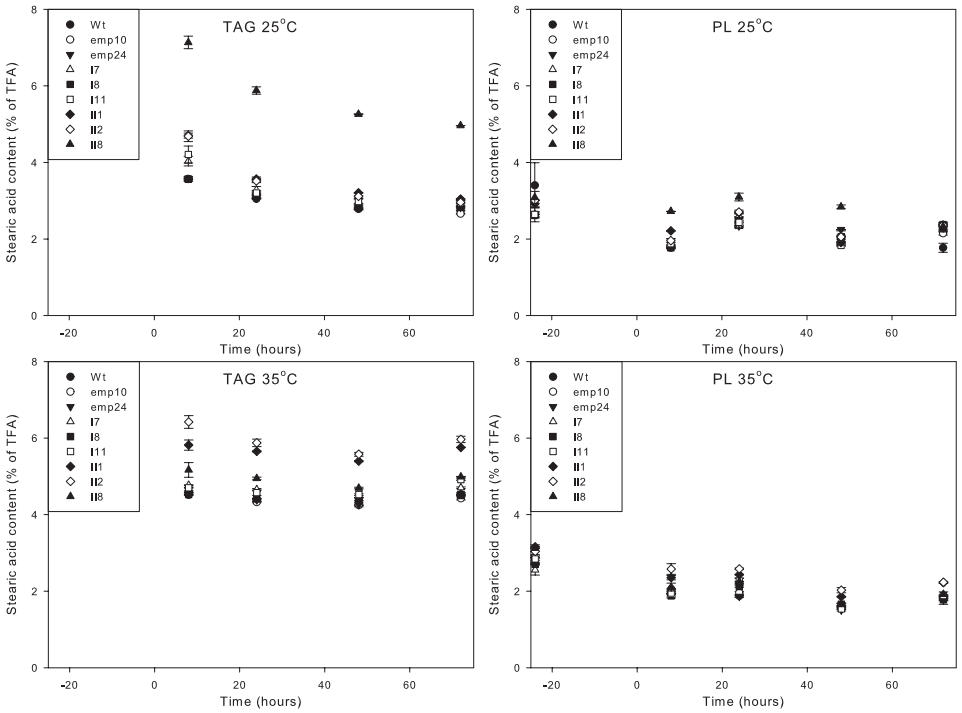

Figure 7.4 Stearic acid content per lipid type for each strain over time. Error bars indicate the distance to the mean of duplicate samples.T-1 is not included in the TAG measurement, since under nitrogen replete conditions TAG contents did not exceed threshold limits. Error bars indicate the distance to the mean between two biological replicates.

The transgenic lines DSI show similar fatty acid profiles to the wild type and there is no effect on the stearic acid content, in contrast to the stearic acid content in the DSII transgenic strains, all three of which showed elevated stearic acid content relative to the wild type. This was particularly evident for DSII8 in which the stearic acid content in the TAG molecules at 25°C was 7.1% compared to 3.6% in the wild type. At 35°C an overall increase in stearic acid content within all strains was found. The wild type accumulated 4.6% stearic acid in the TAG molecules, and the DSII1 and DSII2 strains showed an increase of stearic acid content in TAG molecules up to 5.9% and 6.5% respectively (Figure 7.4). Surprisingly, the DSII8 strain had a lower stearic acid content when grown at 35°C, and the behavior. An explanation for this phenomenon could be that as the silencing construct is randomly integrated into the *C. reinhardtii* genome, this could lead to differences in expression of the construct. Possibly the construct in DSII8 strain was inserted in a region that is more highly expressed at lower temperatures, since DSII8 probably only has one integration site which would make it more susceptible to genomic position effects (Figure 7.1C).

Table 7.3 Fatty acid profiles 8 hours after nitrogen depletion as a percentage of the respective lipid types.

	TAG 25°C									PL 25°C								
	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8
C14:0	0.4*	0.3	0.6	0.4	0.5*	0.3**	0.4**	0.3**	0.5**	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
C16:0	30.7	30.5	30.4	30.6	30.0	30.6	28.0	28.6	30.3	24.7	24.6	24.5	24.5	24.6	24.3	24.4	23.8	24.2
C16:1	6.2	6.4	6.0	6.2	5.8	5.8	6.0	5.7	5.7	6.7	6.9	6.7	6.8	6.5	6.6	6.6	5.9	6.5
C16:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.9	0.9	1.0	1.0	1.3	1.5	1.1
C16:3	1.1	1.0**	1.3	1.6**	2.5*	1.4**	1.3**	1.8**	1.6*	3.0	3.0	3.0	3.0	3.1	3.2	3.2	3.5	3.3
C16:4	2.5	2.7	2.7	2.5	2.7	2.6	3.3**	3.0	3.0	13.9	13.6	13.9	13.7	13.7	13.6	13.0	13.9	14.0
C18:0	3.6	3.6	3.6	4.0	3.6	4.2	4.7	4.7	7.1	1.8	1.8	1.8	1.8	1.8	1.9*	2.2	2.0	2.7
C18:1	39.9	39.1	39.0	38.6	38.0	38.8	36.7	36.3	34.8	22.3	22.3	21.9	22.1	21.8	21.7	21.3	19.7	20.2
C18:2	8.1	8.9	8.3	8.3	8.5	8.9	10.7	10.7	9.1	7.3	7.9	7.8	8.0	8.1	8.4	9.6	9.6	8.2
C18:3	5.3	5.5	5.8	5.2	5.5	5.4	6.5*	6.2	5.7	16.6	16.3	16.7	16.5	16.6	16.5	15.8	17.0	16.9
C18:4	2.2**	2.1	2.3**	2.5**	3.0*	2.0**	2.5*	2.8	2.1**	1.7	1.6	1.7	1.7	1.8	1.7	1.7	2.1**	1.9
SFA	34.7	34.4	34.6	35.1	34.1	35.1	33.2	33.6	37.9	27.5	27.5	27.3	27.3	27.4	27.3	27.7	26.8	27.9
MUSFA	46.1	45.5	45.1	44.7	43.8	44.7	42.7	42.0	40.6	29.1	29.2	28.6	28.8	28.3	28.3	27.8	25.7	26.7
PUFA	19.2	20.2	20.3	20.1	22.1	20.2	24.2	24.4	21.6	43.4	43.3	44.1	43.8	44.3	44.4	44.5	47.5	45.4
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
% SFA/UFA	53.0	52.4	52.9	54.1	51.7	54.1	49.6	50.5	60.9	37.9	37.9	37.6	37.6	37.7	37.5	38.2	36.6	38.8

	TAG 35°C									PL 35°C								
	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8
C14:0	0.5	0.6	0.5	0.6	0.5	0.6	0.6	0.6	0.6	0.7	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
C16:0	26.8	28.0	27.9	27.5	28.0	28.4	26.0	25.3	27.9	24.3	24.1	23.6	23.1	23.1	24.0	24.1	24.4	23.7
C16:1	8.9	8.5	9.0	9.4	9.1	9.0	8.5	8.4	8.9	10.0	9.8	9.9	10.7	10.1	9.7	9.4	9.5	9.9
C16:2	0.6	0.6	0.6	0.4	0.6	0.6	0.8	0.7	0.5	1.3	1.4	1.4	1.1	1.4	1.5	1.7	1.5*	1.3
C16:3	2.2	2.0	2.1	1.9	2.1	2.1	2.2	2.2	2.1	6.0	5.9	6.3	5.9	6.6	6.5	6.1	5.7*	6.5
C16:4	1.5	1.5	1.4	1.4	1.3	1.4	1.5	1.7*	1.3	8.3	8.5	8.7	9.0	9.1	9.2	8.2	8.1*	8.7
C18:0	4.6	4.5	4.6	4.8	4.6	4.8	5.9	6.5	5.3	2.1	1.9	1.9	1.9	1.9	2.0	2.4	2.6*	2.1
C18:1	33.9	33.8	33.8	34.2	33.9	33.6	32.2	31.4	33.3	20.5	19.9	19.6	19.8	19.1	18.1	19.7	20.1*	19.2
C18:2	14.8	14.5	14.5	13.8	14.1	13.5	16.0	17.1	14.0	12.1	12.3	12.3	12.0	12.1	12.3	13.0	12.6	12.0
C18:3	4.8	4.7	4.8	4.8	5.0	5.1	4.8	4.9	4.8	13.8	14.1	14.4	14.4	14.6	14.6	13.5	13.5	14.2
C18:4	1.4**	1.3*	0.9*	1.1*	0.8*	1.0*	1.4**	1.3**	1.2*	0.9*	1.3	1.1*	1.2**	1.2*	1.4**	1.0*	1.3*	1.4
SFA	32.0	33.1	33.0	32.9	33.1	33.8	32.6	32.3	33.7	27.1	26.8	26.3	25.9	25.8	26.7	27.2	27.7	26.6
MUSFA	42.7	42.3	42.7	43.7	42.9	42.6	40.7	39.8	42.2	30.5	29.7	29.5	30.5	29.2	27.8	29.1	29.6	29.1
PUFA	25.3	24.6	24.3	23.4	23.9	23.7	26.7	27.9	24.0	42.5	43.5	44.2	43.6	45.0	45.5	43.7	42.7	44.3
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
% SFA/UFA	47.1	49.5	49.3	49.0	49.5	51.0	48.3	47.7	50.9	37.1	36.6	35.6	34.9	34.8	36.4	37.4	38.3	36.2

All samples, unless otherwise indicated have less than 5% variation between the duplicates

*Samples with 5-10% variation between duplicates

**Samples with >10% variation between duplicates

The DSI silencing construct does not seem to be effective in post transcriptional gene silencing of the SAD gene, since the stearic acid content in the lipids did not change compared to the wild type strain. This could be explained by the fact that the silencing con-

struct DSI is targeting the final exon of the SAD gene and that the successful reduction of this part of the gene translation is not influencing the overall efficiency of the SAD gene.

The stearic acid content is higher in all transgenic strains containing the DSII construct (Figure 7.5). The biggest relative increase in stearic acid is found in strain DSII8 at 25°C, which doubled the stearic acid content in TAG compared to the wild type. Also at 35°C all DSII containing constructs show an increased stearic acid content compared to the wild type, but strains DSII1 and DSII2 show the biggest fold change 1.2 and 1.4 times respectively. The fold change of stearic acid at 35°C is not as high as at 25°C and this can be explained by the fact that the stearic acid content in the wild type strain is already elevated by the higher temperature and that the absolute amount of stearic acid does not exceed the values that are found for the DSII8 strain at 25°C. It is known that at lower temperatures plants and microalgae upregulate the expression of the SAD gene to produce more unsaturated fatty acids to maintain membrane fluidity at lower temperatures (An et al., 2013; Falcone et al., 2004).

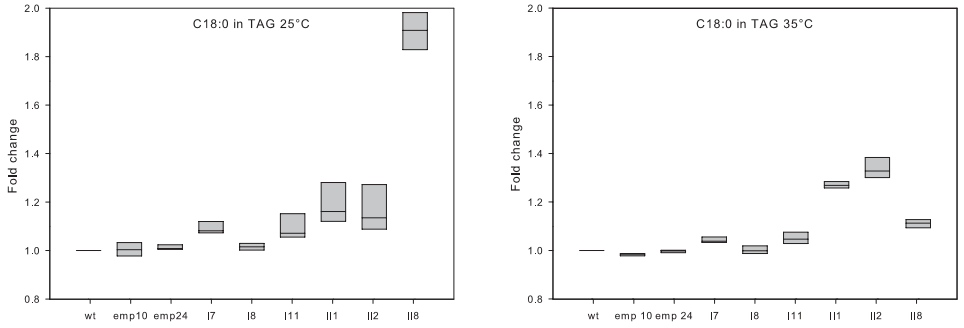

Figure 7.5 Stearic acid content and the fold change in TAG relative to wild type at 25°C and 35°C. The wild type is shown as shown as 1. Gray boxes indicate the spread of the data points throughout the time series and the black line in the box indicates the mean value for each strain.

To assess the level of silencing, the SAD mRNA was quantified using real-time qPCR and compared to that of selected housekeeping genes. It can be seen that the expression of the SAD gene in all strains is similar before the heat shock and thereafter the SAD mRNA levels are reduced in DSII containing strains especially at 25°C (Figure 7.6). This shows that the silencing construct indeed is inducible by a heat shock. The SAD expression seems to be transiently enhanced when the culture experience nitrogen depleted conditions. The increase in SAD expression upon nitrogen depletion, results in the formation of the oleic acid, which is the major constituent of TAG in microalgae. The reduction in activity of this important enzyme for oleic acid production and TAG accumulation could explain the decrease in the TAG content that is found in *C. reinhardtii* when SAD is silenced and the increase in TAG content when SAD is overexpressed (Hwangbo et al., 2014).

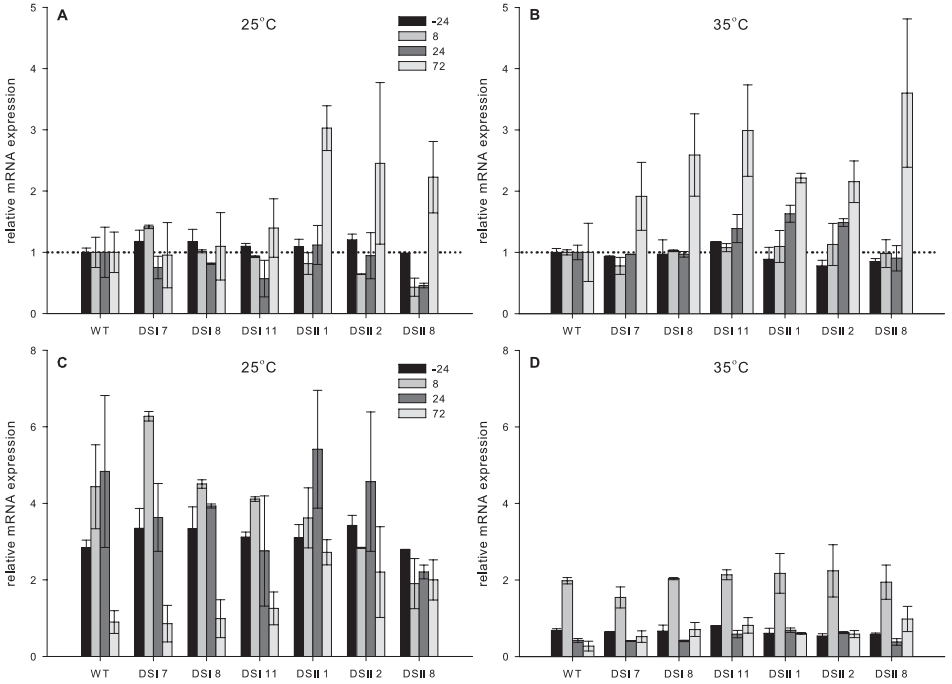

Figure 7.6 Relative mRNA expression of SAD gene in the tested strains and temperature conditions. The mRNA expression values are relative to the housekeeping genes β-tubulin and CBLP. The geographic mean was taken from both housekeeping genes and normalized (set as 1) to the wild type (A and B). The relative mRNA expression values (to housekeeping genes) not normalized to wild type (C and D). Error bars represent standard deviation of biological duplicates.

After 24 hours, the expression starts to decline and after 72 hours the abundance of the SAD mRNA was lower than under nitrogen replete conditions for each strain. At the first two time points after the heat shock, the mRNA levels of the SAD gene in the strains that contain the DSII silencing construct and are grown at 25°C, were reduced. This shows that the silencing construct was indeed able to reduce the mRNA levels of the SAD gene, causing an enrichment of stearic acid in the TAG of *C. reinhardtii*. In Figure 7.7 the stearic acid content in TAG molecules is plotted against the relative SAD mRNA expression. A modest correlation can be found, especially for the 25°C cultures in the beginning of the nitrogen depletion, which indicates that lower mRNA levels for SAD result in higher stearic acid contents in the TAG molecules. It would be interesting to find strains that have even more silencing of SAD, which will potentially have more stearic acid in their TAG molecules. The silencing at 35°C is less efficient and this can be caused by the temperature effect, since SAD expression is higher at lower temperatures (James et al., 2013). The DSII containing strains show a relatively higher SAD expression at the end of the experiment and the silencing effect has faded. It could be that the DSII containing

transformants are overexpressing SAD to compensate for the higher stearic acid content in the cells. The DSI containing constructs do not show a different SAD expression compared to the wild type and this corresponds with the unchanged stearic acid content in the DSI containing strains.

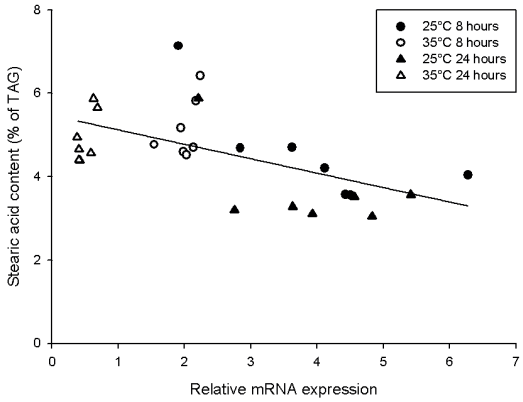

Figure 7.7 Correlation between SAD relative expression and stearic acid content in TAG. The results are shown for the first two time points after nitrogen depletion and silencing induction, 8 hours (circles) and 24 hours (squares). Blue and red symbols represent the results for growth at 25°C and 35°C respectively.

In the beginning of the nitrogen depletion phase and the expression phase of the silencing construct, the stearic acid content is quickly increased in the DSII containing transgenic lines. The percentage of stearic acid in TAG lipids decreases after the first 24 hours, indicating that the silencing of the SAD gene is most likely most active during the first hours, which is supported by the SAD expression data (Figure 7.6). This could indicate that in the beginning, when silencing is strongest, C18:0 is integrated into TAG molecules and cannot be converted afterwards when SAD silencing is reduced. The C18:0 content does not increase much subsequently, because SAD is less silenced. In the study of Hwangbo et al. the SAD gene was overexpressed in wild type *C. reinhardtii* cells and they found an increase of oleic acid content compared to the wild type (Hwangbo et al., 2014). They did not observe a decrease in stearic acid content however. This would suggest that the content of stearic acid is tightly regulated and that the increased demand for oleic acid does not result in an increased stearic acid pool. In addition, the stearic acid content was maintained at a constant level for all strains tested under different conditions (Hwangbo et al., 2014).

Under normal growth conditions, the stearoyl-ACP molecule is generally rapidly desaturated by SAD to form oleoyl-ACP inside the chloroplasts resulting in low presence of stearic acid in glycerolipids. The further desaturation or elongation of oleoyl-ACP into

polyunsaturated fatty acids (PUFAs) can occur in either the chloroplast using the prokaryotic pathway (Roughan et al., 1980), or the acyl-ACP molecules are exported outside the chloroplast as acyl-CoA molecules and enter the eukaryotic pathway (Dubacq et al., 1976; Simpson and Williams, 1979; Slack and Roughan, 1975; Slack et al., 1976) taking place in the endoplasmic reticulum (Figure 7.8). The steps towards PUFAs, are catalyzed by membrane bound enzymes in contrast to the soluble SAD, resulting in membrane glycerolipids or triacylglycerol of 16 to 20 carbon chain lengths long with various degrees of saturation. Over the past decades many SAD genes have been identified in a broad range of organisms and often these genes encode different isoforms (Byfield et al., 2006; Kachroo et al., 2007). These isoforms can differ from the original SAD in the location where double bonds are introduced or they have alternative substrate length specificity (Cahoon et al., 1997). The increase in the stearic acid content resulting from the transient silencing of the SAD gene is significant, but not as pronounced as has been observed in SAD mutants in plants and other organisms. A reason for this could be the presence of SAD isoforms in *C. reinhardtii*. However, there has been no evidence for other soluble SAD genes in the genome of *C. reinhardtii*. In *Arabidopsis,* expression of none of the six isozymes could recover the SAD deficient mutant phenotype (Kachroo et al., 2007), indicating that these isoforms have a different function in this plant and supporting the hypothesis that there is just a single enzyme that has the SAD properties.

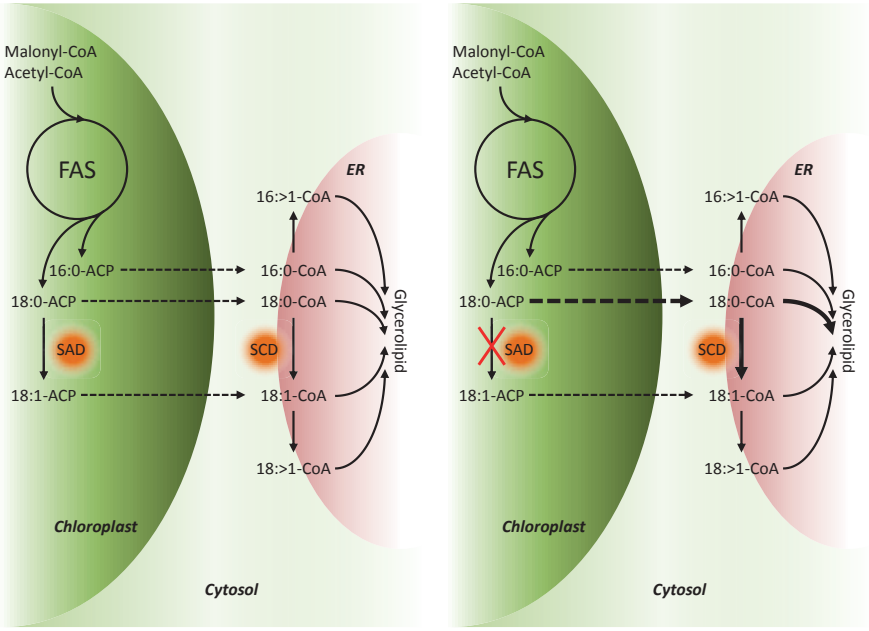

Figure 7.8 Schematic representation of the fatty acid synthesis and desaturation in the chloroplast and endoplasmic reticulum. FAS: Fatty Acid Synthesis. SAD: Stearoyl-ACP Desaturase. SCD: Stearoyl-CoA Desaturase. ER: Endoplasmic Reticulum.

Plants are able to accumulate much more stearic acid than microalgae (Table 7.1). Note that these plants are all from areas with high temperatures, which enable the accumulation of higher saturated fatty acid contents. An explanation could be the different biological function of lipid storage in plant seeds compared to TAG in microalgae. Another reason could be that the compartmentalization of microalgae is causing the difference in stearic acid over-accumulation. Potentially the stearoyl-ACP is transported out of the chloroplast and the fatty acid is desaturated in the ER by another enzyme that is not affected by the silencing (Figure 7.8). The function of TAG in oleaginous microalgae is likely to be very different from the TAG that is accumulated and stored in plant seeds, and more research is needed to understand the role and mechanisms of TAG. Another bottleneck could be that the enzyme that liberates the acyl-ACP chain from the fatty acid synthesis cycle is poorly expressed or has a low affinity for stearoyl-ACP. Similarly the enzymes that incorporate the acyl chain in the glycerolipids might have low affinity for stearoyl chains compared to other fatty acids such as oleoyl. There are unicellular organisms found that can accumulate high amounts of intracellular stearic acid in (Ratledge and Cohen, 2010; Ykema et al., 1989), which is proof that stearic acid rich TAG molecules in microorganisms are feasible.

To increase the stearic acid content of the TAG molecules in *C. reinhardtii* further, the cultures could be exposed to several heat shocks over time to maintain expression of the silencing construct. Although we expected that growing the transgenic lines at 35°C would result in a continuous expression of the silencing construct because it is very close to the heat shock temperature (40°C). This is not the case, probably because the heat shock protein needs a transient big shift in temperature to be active and not a constant high temperature. Furthermore, the higher temperature is another stress factor for the microalgae, explaining the cultures' loss of viability after five days of nitrogen depletion at 35°C.

Another option would be to also reduce the mRNA level of the Stearoyl-CoA Desaturase (SCD) enzyme, which can desaturate the stearoyl-CoA acyl chain which is present outside the chloroplast (Figure 7.8). It is very likely that the accumulated stearic acid is converted to oleic acid in the cytosol and endoplasmic reticulum. In *C. reinhardtii* the SCD is not well annotated: there is a predicted protein sequence for this enzyme and therefore a more thorough study needs to be done on characterizing and annotating the desaturase genes involved in the desaturation of fatty acids outside the chloroplasts. Another possible way to reduce the enzyme function of the SCD would be by using inhibiting fatty acids that are known to reduce enzyme efficiency of specific desaturases. The cyclic inhibitor sterculic acid could be used to reduce the SCD efficiency (Cao et al., 1993; James et al., 1968; Moreton, 1985; Wältermann and Steinbüchel, 2000). This could be a potential way to reduce overall stearoyl desaturase effectiveness in combination with

amiRNA gene silencing, to study the effect of stearic acid accumulation in microalgae. It is possible that doing this will reduce the viability of the cells, since they cannot cope with the increased stearic acid levels.

7.4 Conclusion

Artificial microRNA was used to reduce the stearoyl-ACP desaturase activity in *C. reinhardtii*. The mRNA expression was transiently reduced upon the induction of the amiRNA construct by heat shock and nitrogen depleted growth conditions, which doubled the stearic acid content in *C. reinhardtii*. The overall stearic acid content could be further increased by blocking the conversion of stearoyl to oleoyl in other compartments. Optionally the enzyme activity can be reduced by using specific cyclopropene inhibitors, to circumvent silencing the poorly annotated SCD gene. Furthermore, the next step should be silencing both SAD and SCD genes in oleaginous microalga, since the TAG accumulating mechanisms are more developed in these species and possibly the response to SAD silencing is different from *C. reinhardtii*. Alternatively, oleaginous microalgae that are isolated from high temperature ecosystems should be used, since these species normally contain a higher saturated fatty acid content.

Acknowledgements

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Appendix

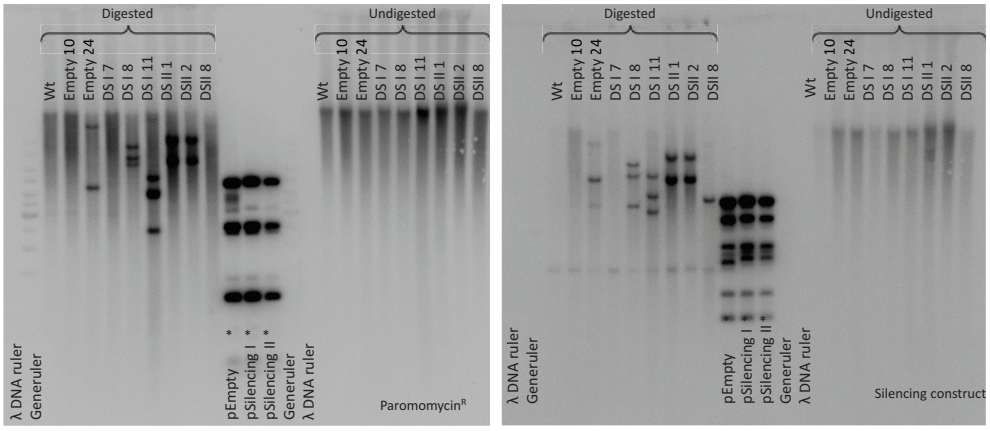

Figure 7.A1 Southern blots showing both the XmnI digested and undigested DNA blotted with either the paromomycin probe (left) and the ami insert probe (right).

Table 7.A1 Fatty acid profile in TAG and total TAG content as percentage of cell dry weight. Error values represent the standard deviation between the biological replicates

25°C		Fatty acid										Total
Time	Strain	C14:0	C16:0	C16:1	C16:2	C16:3	C16:4	C18:0	C18:1	C18:2	C18:3	C18:4	TAG
T1	Wt	0.02 ± 0.00	1.64 ± 0.05	0.33 ± 0.01	0.00 ± 0.00	0.06 ± 0.00	0.13 ± 0.00	0.19 ± 0.00	2.13 ± 0.07	0.43 ± 0.02	0.28 ± 0.01	0.12 ± 0.02	5.3
	DSII1	0.02 ± 0.01	1.09 ± 0.00	0.23 ± 0.00	0.00 ± 0.00	0.05 ± 0.01	0.13 ± 0.02	0.18 ± 0.00	1.42 ± 0.00	0.41 ± 0.01	0.25 ± 0.03	0.10 ± 0.01	3.9
	DSII2	0.01 ± 0.01	1.02 ± 0.01	0.20 ± 0.00	0.00 ± 0.00	0.06 ± 0.02	0.11 ± 0.00	0.17 ± 0.01	1.29 ± 0.00	0.38 ± 0.01	0.22 ± 0.00	0.10 ± 0.01	3.6
	DSII8	0.02 ± 0.01	1.10 ± 0.03	0.21 ± 0.01	0.00 ± 0.00	0.06 ± 0.00	0.11 ± 0.00	0.26 ± 0.01	1.26 ± 0.04	0.33 ± 0.02	0.21 ± 0.00	0.08 ± 0.01	3.6
T2	Wt	0.10 ± 0.00	6.80 ± 0.40	1.20 ± 0.00	0.10 ± 0.00	0.60 ± 0.00	0.70 ± 0.00	0.70 ± 0.00	6.90 ± 0.30	3.10 ± 0.10	1.70 ± 0.10	0.35 ± 0.05	22.3
	DSII1	0.10 ± 0.00	5.25 ± 0.05	1.05 ± 0.05	0.10 ± 0.00	0.70 ± 0.00	0.95 ± 0.05	0.70 ± 0.00	5.75 ± 0.05	3.55 ± 0.05	1.55 ± 0.05	0.40 ± 0.00	20.1
	DSII2	0.10 ± 0.00	5.50 ± 0.10	1.05 ± 0.05	0.10 ± 0.00	0.75 ± 0.05	1.00 ± 0.00	0.70 ± 0.00	5.75 ± 0.15	3.85 ± 0.05	1.70 ± 0.00	0.40 ± 0.00	20.9
	DSII8	0.10 ± 0.00	6.35 ± 0.35	1.10 ± 0.00	0.10 ± 0.00	0.65 ± 0.05	0.75 ± 0.05	1.25 ± 0.05	5.65 ± 0.15	2.95 ± 0.15	1.65 ± 0.15	0.35 ± 0.05	20.9
T3	Wt	0.08 ± 0.01	9.21 ± 0.21	1.63 ± 0.02	0.37 ± 0.01	1.13 ± 0.01	1.27 ± 0.02	0.92 ± 0.02	9.36 ± 0.19	5.86 ± 0.13	2.77 ± 0.05	0.34 ± 0.01	32.9
	DSII1	0.07 ± 0.00	8.40 ± 0.15	1.28 ± 0.00	0.50 ± 0.02	1.03 ± 0.03	1.25 ± 0.05	0.99 ± 0.02	7.79 ± 0.11	6.28 ± 0.14	2.52 ± 0.08	0.36 ± 0.01	30.5
	DSII2	0.07 ± 0.00	8.41 ± 0.50	1.18 ± 0.10	0.48 ± 0.03	1.04 ± 0.07	1.24 ± 0.08	0.95 ± 0.05	7.58 ± 0.49	6.35 ± 0.39	2.58 ± 0.14	0.38 ± 0.02	30.3
	DSII8	0.07 ± 0.00	8.30 ± 0.29	1.39 ± 0.02	0.35 ± 0.02	1.09 ± 0.03	1.18 ± 0.04	1.58 ± 0.05	7.58 ± 0.21	5.20 ± 0.16	2.58 ± 0.10	0.33 ± 0.00	29.7
T4	Wt	0.06 ± 0.01	10.52 ± 0.29	1.64 ± 0.05	0.53 ± 0.01	1.33 ± 0.04	1.51 ± 0.03	1.02 ± 0.03	9.64 ± 0.30	6.98 ± 0.17	3.19 ± 0.07	0.31 ± 0.00	36.7
	DSII1	0.05 ± 0.00	10.19 ± 0.23	1.42 ± 0.01	0.79 ± 0.03	1.30 ± 0.04	1.57 ± 0.06	1.12 ± 0.02	8.57 ± 0.17	8.01 ± 0.23	3.15 ± 0.11	0.36 ± 0.01	36.5
	DSII2	0.05 ± 0.00	9.90 ± 0.18	1.29 ± 0.00	0.71 ± 0.01	1.24 ± 0.01	1.49 ± 0.02	1.04 ± 0.03	8.09 ± 0.09	7.75 ± 0.09	3.03 ± 0.03	0.37 ± 0.01	35.0
	DSII8	0.07 ± 0.02	10.65 ± 0.09	1.61 ± 0.00	0.57 ± 0.01	1.44 ± 0.00	1.56 ± 0.01	1.87 ± 0.01	8.99 ± 0.07	6.98 ± 0.05	3.30 ± 0.01	0.33 ± 0.00	37.4
35°C		Fatty acid										Total
Time	Strain	C14:0	C16:0	C16:1	C16:2	C16:3	C16:4	C18:0	C18:1	C18:2	C18:3	C18:4	TAG
T1	Wt	0.05 ± 0.00	2.34 ± 0.09	0.77 ± 0.03	0.05 ± 0.00	0.19 ± 0.01	0.13 ± 0.01	0.41 ± 0.01	2.96 ± 0.10	1.29 ± 0.06	0.42 ± 0.02	0.12 ± 0.01	8.7
	DSII1	0.05 ± 0.00	2.25 ± 0.21	0.73 ± 0.06	0.07 ± 0.01	0.19 ± 0.01	0.13 ± 0.01	0.51 ± 0.02	2.78 ± 0.17	1.38 ± 0.06	0.42 ± 0.04	0.12 ± 0.00	8.6
	DSII2	0.05 ± 0.01	2.13 ± 0.29	0.71 ± 0.08	0.06 ± 0.01	0.19 ± 0.01	0.14 ± 0.01	0.54 ± 0.04	2.64 ± 0.32	1.43 ± 0.09	0.41 ± 0.05	0.11 ± 0.00	8.4
	DSII8	0.06 ± 0.00	2.72 ± 0.26	0.87 ± 0.09	0.05 ± 0.01	0.21 ± 0.01	0.13 ± 0.00	0.51 ± 0.03	3.25 ± 0.30	1.36 ± 0.12	0.47 ± 0.05	0.12 ± 0.00	9.8
T2	Wt	0.06 ± 0.00	4.83 ± 0.03	2.09 ± 0.06	0.11 ± 0.00	0.55 ± 0.02	0.35 ± 0.01	0.86 ± 0.01	6.17 ± 0.13	3.39 ± 0.09	0.92 ± 0.03	0.15 ± 0.00	19.5
	DSII1	0.06 ± 0.00	4.18 ± 0.08	1.79 ± 0.05	0.15 ± 0.00	0.57 ± 0.02	0.36 ± 0.01	1.01 ± 0.02	5.19 ± 0.14	3.32 ± 0.08	0.83 ± 0.02	0.17 ± 0.00	17.6
	DSII2	0.06 ± 0.00	4.24 ± 0.16	1.89 ± 0.06	0.14 ± 0.00	0.56 ± 0.02	0.37 ± 0.01	1.09 ± 0.06	5.23 ± 0.18	3.52 ± 0.13	0.85 ± 0.02	0.20 ± 0.04	18.2
	DSII8	0.06 ± 0.00	4.35 ± 0.15	1.96 ± 0.10	0.10 ± 0.00	0.52 ± 0.02	0.33 ± 0.02	0.89 ± 0.03	5.52 ± 0.22	3.10 ± 0.12	0.80 ± 0.04	0.19 ± 0.01	17.8
T3	Wt	0.06 ± 0.00	7.82 ± 0.01	3.13 ± 0.03	0.20 ± 0.01	0.96 ± 0.00	0.74 ± 0.00	1.31 ± 0.02	8.78 ± 0.08	5.23 ± 0.00	1.80 ± 0.01	0.23 ± 0.00	30.3
	DSII1	0.07 ± 0.01	7.49 ± 0.10	2.84 ± 0.07	0.34 ± 0.00	0.98 ± 0.02	0.71 ± 0.00	1.59 ± 0.02	7.93 ± 0.18	5.39 ± 0.08	1.71 ± 0.02	0.21 ± 0.01	29.3
	DSII2	0.06 ± 0.00	7.75 ± 0.40	3.07 ± 0.12	0.33 ± 0.02	1.02 ± 0.06	0.76 ± 0.06	1.74 ± 0.08	8.18 ± 0.28	5.80 ± 0.38	1.88 ± 0.21	0.25 ± 0.02	30.9
	DSII8	0.06 ± 0.00	7.85 ± 0.12	3.19 ± 0.02	0.20 ± 0.01	0.96 ± 0.01	0.71 ± 0.01	1.43 ± 0.01	8.62 ± 0.02	5.14 ± 0.09	1.81 ± 0.11	0.24 ± 0.00	30.2
T4	Wt	0.07 ± 0.00	8.92 ± 0.08	3.39 ± 0.03	0.27 ± 0.00	1.21 ± 0.01	1.03 ± 0.02	1.59 ± 0.01	9.54 ± 0.15	6.10 ± 0.02	2.31 ± 0.05	0.31 ± 0.01	34.7
	DSII1	0.07 ± 0.00	8.74 ± 0.27	3.12 ± 0.05	0.46 ± 0.02	1.27 ± 0.02	0.99 ± 0.03	1.98 ± 0.05	8.76 ± 0.16	6.33 ± 0.19	2.12 ± 0.09	0.29 ± 0.01	34.1
	DSII2	0.07 ± 0.00	8.64 ± 0.10	3.21 ± 0.08	0.41 ± 0.01	1.25 ± 0.02	0.98 ± 0.00	2.05 ± 0.00	8.57 ± 0.14	6.39 ± 0.06	2.13 ± 0.03	0.32 ± 0.00	34.0
	DSII8	0.06 ± 0.00	8.70 ± 0.21	3.36 ± 0.05	0.26 ± 0.01	1.21 ± 0.03	0.97 ± 0.02	1.69 ± 0.03	9.14 ± 0.09	5.73 ± 0.12	2.13 ± 0.08	0.30 ± 0.01	33.6

Chapter 8

General Discussion

Despite the fact that microalgae have been the subject of many research programs in the past years, the number of microalgae derived products on the market is still limited. One of the problems causing this discrepancy is that the microalgal strains used are not yet optimized to be applied in industrial processes. In this chapter, the results from the work described in this thesis, strain improvement and bottlenecks in genetic engineering of microalgae, are discussed. Furthermore, several metabolic engineering targets are discussed, which could enable the successful realization of industrial microalgal production strains in the future. Bottlenecks in genetic engineering will not only be of technical or biological origin, but will also be related to public acceptance and legislation concerning genetically modified microalgae.

8.1 Introduction

Microalgae are an alternative sustainable renewable production crop, which do not need arable land for cultivation. Furthermore, the need for fresh water can be reduced when salt tolerant microalgae are used. The outlook for microalgae derived commodities in the future is therefore promising (Breuer et al., 2013b; Chisti, 2007; Wijffels and Barbosa, 2010). Although the expected biomass and oil productivity in microalgae is much higher than in agricultural oil crops (Chisti, 2007), the production costs are still too high for microalgal derived oils to be economically feasible. The cost- price of microalgal products can be reduced by either reducing the costs of microalgal production, or by increasing the productivity in microalgae further. This in turn can be achieved with smart engineering solutions and physiological adaptations of microalgal production strains. Increasing the productivity of microalgae will largely depend on metabolic engineering strategies. The solar energy fixed by photosynthesis is stored as chemical energy in a wide variety of molecules and not all of these molecules are relevant for industry. With metabolic engineering we intend to increase the yield on light with respect to desired products. The challenges that need to be overcome, before metabolic engineering can be applied on industrially relevant microalgal strains, will be discussed.

8.2 Major bottlenecks in strain improvement and the future of genetic engineering of microalgae

Neutral lipids, such as triacylglycerol (TAG) are an example of a potential microalgal derived product, which could be applied for edible oils as well as for biodiesel production. Most microalgal studies on metabolic engineering and lipid biosynthesis have been done with a limited number of model strains, of which *Chlamydomonas reinhardtii* is the most widely used model species for microalgal research. Decades of research on photosynthesis have resulted in a wide range of metabolic engineering tools and a well annotated genome (Mayfield et al., 2007; Merchant et al., 2007; Shimogawara et al., 1998) for this species. Although extensive biological insight and a genetic engineering toolbox is a clear advantage, *C. reinhardtii* accumulates only large amounts of starch upon nitrogen depletion and does not accumulate triacylglycerol under these conditions. This issue was resolved by using mutants that lack the ability to produce starch and therefore increase their TAG content 10-fold up to 20% of cell dry weight (Li et al., 2010a; Zabawinski et al., 2001). However, these strains are not robust, as they exhibit a growth reduction of 30% and their primary metabolism still depends on the presence of starch, to obtain the required energy by respiration during the night. Furthermore, known oleaginous microalgal strains are already capable of producing much higher amounts of TAG under nitrogen starvation. Unfortunately the toolbox for metabolic engineering of oleaginous algae is still in its infancy (**Chapter 1 and 6**). Ideally we would like to obtain an oleaginous

microalgal cell factory which is able to channel almost the entire carbon and energy flow towards TAG accumulation.

Translation of metabolic engineering strategies from one species to the other is very difficult. The genetic toolbox cannot be used for all species because the genome of the different microalgae phyla are extremely diverse (Hoek, 1995). The way to introduce DNA in algae will be different per species as well, because the cell wall is very distinct for different species. Some species are known to have a glycoprotein based cell wall such as *C. reinhardtii* (Macfie et al., 1994; Voigt, 1988). Other species, such as *Dunaliella,* completely lack a cell wall (Oren, 2005), or have complex cellulose based cell walls such as *Chlorella* species (Pieper et al., 2012), or have very resistant cell walls containing algaenan, such as *Scenedesmus* or *Nannochloropsis* species (Burczyk et al., 1970; Burczyk et al., 1999; Scholz et al., 2014). The focus should preferably be on a small selection of industrially relevant microalgae, to enable the development of efficient metabolic engineering tools and strategies, which has been the successful approach used in industrial development of bacterial and yeast strains.

In **chapter 6** we propose a generic method that can be used to optimize parameters for transforming microalgal species that do not yet have a well-established transformation protocol. Such a method is very useful in developing successful genetic engineering tools for less well-characterized microalgae, such as most oleaginous microalgae, for which the development of transformation protocols is a bottleneck. The use of two fluorophores enables the study of cell permeability which is a proxy for the potency to take up exogenous plasmid DNA through the micro-pores introduced by an electric pulse. Subsequently, a second fluorescent dye can be used to determine the cell viability after recovery from the electric pulse. This fast screening method can be used to test different parameters and determine the most optimal transformation settings for each microalga specifically.

8.3 Mutagenesis approach for strain improvement

In case direct genetic engineering strategies cannot be applied, the generation of a mutant library with improved properties could be an option. Mutagenesis is based on exposing the species of interest to a mutagenic source to induce random mutations in the genome, which can, for example, reduce enzyme efficiency or completely knockout the gene function. Mutagens that are frequently used are UV irradiation, which causes transversions in the genome, or chemical mutagens such as ethidium bromide (intercalate DNA), ethyl methanesulfonate (Lee et al., 2014), and methylnitronitrosoguanidine. A disadvantages of mutagenesis is that comprehensive screening is required to select the mutants with the desired phenotype.

Another method to generate mutants is insertional mutagenesis, in which a selection marker is randomly introduced in the host genomic DNA. When this selection marker is introduced in a gene, the gene will code for a non-functional product. The exact mutation can be identified simply by PCR, because the sequence of the selection marker is known. The disadvantages of insertional mutagenesis are that it can only be used when an efficient transformation protocol is available, it results predominantly in loss of function mutations and the mutants are considered as genetically modified organisms (GMOs).

The oleaginous microalgae *Scenedesmus obliquus* has great potential as a lipid production organism. It has a high lipid productivity when lipid accumulation is induced (Breuer et al., 2014; de Jaeger et al., 2014). In addition to triacylglycerol, *S. obliquus* also accumulates starch in the early phase of nitrogen depletion (**Chapter 4**). We hypothesized that if we could block the starch synthesis in *S. obliquus*, the C3 sugars produced in the Calvin cycle that would normally go towards starch accumulation would then be used for the production of triacylglycerol. Since there was no transformation approach for *S. obliquus* available, UV-mutagenesis was used as is described in **chapter 3**. The introduction of the mutations is random, so a strong selection or screening method to find the mutant of interest is needed. In the case of the starchless mutant we were able to screen the mutant library for the presence of starch. We stained the starch with Iodine vapor and looked for mutants that did not turn purple, by exposing the cultures on nitrogen depleted medium, which induced starch accumulation in the wild type and simultaneously bleached the chlorophyll of the strains to improve the visibility of the staining method. We found 6 mutants with lowered starch content and we selected the best performing starchless mutant (Slm1) (**Chapter 3**). The TAG productivity was increased by over 35% in the Slm1 strain (0.359±0.008 g TAG l^{-1} day^{-1}) compared to the wild type (0.265±0.004 g TAG l^{-1} day^{-1}), and the maximal TAG content went from 45±1% to 57±0.2% (**Chapter 4**). The mutation was found by RNA sequencing of both the wild type and the Slm1 strains under nitrogen replete conditions (**Chapter 5**) which revealed a point mutation in the small subunit of the ADP-glucose pyrophosphorylase gene where an adenine (A) was changed to a thymine (T), which resulted in an amino acid change. The Lysine amino acid (AAG) in the wild type changed to a STOP codon (TAG) in the Slm1 strain. This results in a non-functional small subunit of ADP-glucose pyrophosphorylase (EC:2.7.7.27), and therefore in a knock out of the enzyme. Interestingly, the same gene was mutated in the BAFJ5 starchless mutant of *C. reinhardtii* (Zabawinski et al., 2001).

It is very interesting to see that there is such a different effect of blocking starch synthesis in different microalgal species. In *C. reinhardtii*, the cells are not able to survive day night cycles anymore, since the starch is needed for supply of the necessary carbon and energy during the dark period. *S. obliquus* Slm1 cells were shown to survive day night cycles, and we suggest that the Slm1 strain is respiring TAG to liberate carbon and ener-

gy that is needed during the night. An interesting study would be to follow the biomass composition of *S. obliquus* starchless mutants during the day and night, to see whether TAG is respired in the dark. In addition to studying the TAG content, other potential storage carbohydrates than starch should also be analyzed. This will give information about the adaptation in physiology of the Slm1 strain to cope with the absence of starch and possibly lead to further targets for improving the lipid productivity. The robustness and behavior of the Slm1 strain has proven to be comparable to the wild type strain under lab conditions. However, both cultures should be compared under outdoor conditions as well, since the Slm1 mutant should also be able to resist fluctuating temperatures, light intensities, and possible contaminating microorganisms.

Another experiment that should be executed is studying the effect of the targeted knockdown of TAG catabolism. A potential target enzyme to knock down would be the multifunctional lipase/phospholipase/acyltransferase, which resulted in higher lipid yields in the diatom *Thalassiosira pseudonana* (Trentacoste et al., 2013). The knockdown of starch synthesis in combination with the knockdown of TAG catabolism would be very interesting to study in the starchless mutants of *S. obliquus*. Potentially the TAG productivity will increase even more, although the cells might not be able to survive day night cycles. A possible solution for this problem would be to shift the culture from autotrophic to heterotrophic growth during the night, to supplement the cells with sufficient energy and carbon. Another option would be to supplement the culture with artificial light to maintain just enough photosynthetic activity for maintenance at night. Although the use of additional electricity for lighting would have implementation for the sustainability and cost of the final process.

8.4 Development of more competent cell lines

In **chapter 6** it was found that *Nannochloropsis sp.* and *S. obliquus* are very resistant to electroporation, and harsh settings are needed to introduce pores in the cell membrane to enable DNA uptake. The cell walls of these species are complex with several layers of different molecule structures. *S. obliquus* has an inner layer of cellulose and around this cellulose layer is a trilaminar structure which is known as algaenan (Allard et al., 1998; Burczyk et al., 1970; Burczyk et al., 1999). The cell wall of another *Nannochloropsis* species, *Nannochloropsis gaditana* (strain CCMP 526), has a similar organization to *S. obliquus*, a cellulosic inner wall and a hydrophobic algaenan outer layer (Scholz et al., 2014).

The extracellular algaenan layer is extremely resistant as no degrading enzymes or solvents have been described, which prevents the formation of protoplasts to increase electroporation efficiency in these microalgae. Protoplasts are cells from which the cell

wall has been removed using enzymes or solvents, resulting in very sensitive, but competent cells for transformation. It has been observed that *S. obliquus* cells that were engulfed by grazers, could not be digested and escaped the grazers unharmed, which clearly illustrates the evolutionary benefit of a strong cell wall. In large scale production processes a strong cell wall is desired as well to increase resistance to shear stress, which is likely to occur in large scale bioreactors (Michels et al., 2015).

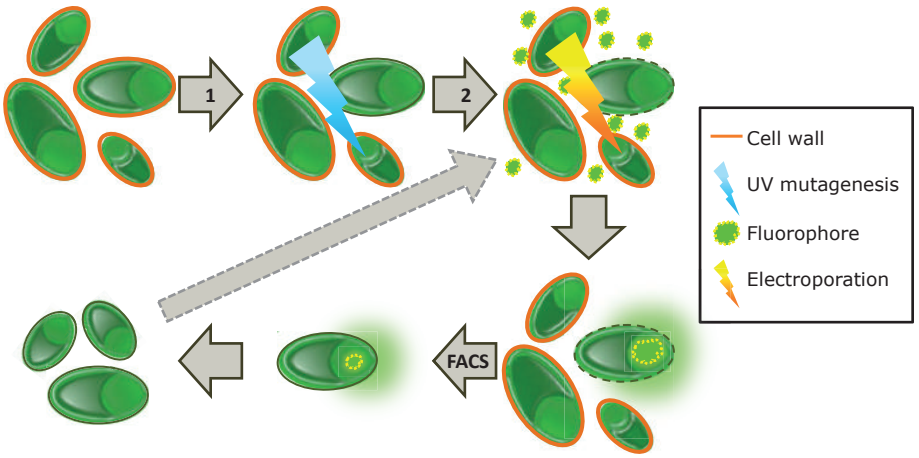

Figure 8.1 Method to create a more competent cell line for microalgae that are difficult to transform or break open for extraction of biomolecules. Step 1 UV mutagenesis. Step 2 Electroporation in the presence of fluorophore. FACS: Fluorescence activated cell sorting.

To overcome transformation problems in strains with resistant complex cell walls, we propose to create a more competent cell line, by using UV mutagenesis in combination with fluorophores and fluorescent activated cell sorting. When electroporation experiments are attempted on robust strains, such as *S. obliquus*, the resistant cell wall inhibits the successful introduction of micro-pores that can enable the exogenous DNA to enter the cells. Alternatively, mutants could be generated by exposing the microalgal cell population to UV irradiation. The UV treated cells are recovered, cultured for a few days, and serially diluted to remove non-viable cells. Subsequently, the cells could be electroporated in the presence of a water soluble fluorophore, impermeant to live cells (such as: Sytox Green, Propidium iodide, YOYO-1, 7-AAD), and the more competent cells are able to take up the fluorophore. Next, cells are recovered from the electric pulse and subsequently the cells that have taken up the fluorophore are sorted in a flow cytometer coupled with a Fluorescent Activated Cell Sorter (Figure 8.1). The cycle including electroporation and cell sorting could be repeated to remove false positives from the mixture. In this repetitive cycle, the electroporation settings, such as the voltage, could be reduced to enrich the culture for more competent cells. To obtain an axenic culture

the mutants with an increased competence for electroporation could be plated and single colonies obtained and screened for other characteristics such as growth rate, and biomolecule extractability.

8.5 Metabolic engineering of microalgae in future applications

The tools that will be developed to enable the short term higher values products will enable genetic engineering of oleaginous microalgae for the production of commodity products in the future. These tools will also enable modifications of physiological traits such as photosynthesis, cell wall composition and temperature tolerance. Once genetic engineering tools are in place, the development of industrially relevant production

Table 8.1 Prospects of microalgal production strains

Target trait/technology	Category	Comment
Algaenanless mutants	Metabolic Engineering	Increase transformability and ease downstream processing (DSP)
Transformation protocols	Metabolic Engineering	Transformation protocols available for all strains
CRISPR/Cas9 system	Metabolic Engineering	Enables direct gene knock out
Target gene products to specific subcellular locations	Metabolic Engineering	Enabling more targeted expression
Mitigating technology	Metabolic Engineering	Needed for GMO production at large scale
Increased lipid yield	(Lipid) Productivity	Higher Productivity
Maintain photosynthetic efficiency under nitrogen depletion	(Lipid) Productivity	Higher Productivity
Increased carbon fixating mechanism	(Lipid) Productivity	Higher Productivity
Increased photosynthetic efficiency	(Lipid) Productivity	Higher productivity
Simultaneous growth and lipid production	(Lipid) Productivity	No need for two-step process
Inducible auto-flocculation	Cost reduction	Reduction in DSP costs
Product secretion (lipid)	Cost reduction	Reduction in DSP costs, and continuous cultivation
Increase salt tolerance	Cost reduction	Reduction fresh water footprint
Nitrogen fixation	Cost reduction	Reduction of fertilizer
Resistance to grazers	Robustness	More robust in outdoor cultivation
Microalgal-microbe communities	Robustness	More robust in outdoor cultivation
High temperature tolerance	Robustness	More robust in outdoor cultivation
Increased oxygen resistance	Robustness	More robust in outdoor cultivation

strains will be feasible. In this chapter we focus on lipids as the target product, but many of the strategies will apply to other products of interest as well. In table 8.1 different focus areas are defined with regard to targets to be engineered into microalgae. Some examples of the four main categories are discussed in more detail below. To realize an economically feasible microalgal lipid production platform, the following strain improvement strategies are needed: development of state of the art metabolic engineering tools, increasing lipid productivity, cost reduction, and creating more robust production strains.

8.5.1 Metabolic engineering tools

The first category of target traits that should be engineered in microalgae are the traits that result in improved metabolic engineering. The example of the more competent cell line has been discussed earlier in this chapter and the improved transformation protocols are covered in **chapter 6**. Furthermore, regulatory sequences, such as promoter, intron, and terminator sequences, which enable stable expression of transgenes in target species, should be developed (**Chapter 1**). The efforts on metabolic engineering of microalgae are increasing, which results in the development of new DNA delivery methods and other metabolic engineering techniques on a regular basis (Daboussi et al., 2014; Kim et al., 2014).

8.5.1.1 CRISPR/CAs9

Since homologous recombination, which is needed to knock out genes, is very inefficient in microalgae (Gumpel et al., 1994; Sodeinde and Kindle, 1993), researchers relied on RNA silencing for knocking down genes. With the recent discovery of genome editing systems, such as zinc finger nucleases, TALEN and CRISPR/Cas9 technology (Jinek et al., 2012), there is the potential to specifically knock out genes, enabling editing of the nuclear genome of many organisms (Belhaj et al., 2015; DiCarlo et al., 2013; Jiang et al., 2013; Qin et al., 2015). The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat) system is a prokaryotic immune system that targets foreign genetic elements and provides a form of acquired immunity. An RNA-template, called single guide RNA (sgRNA), can recognize specific DNA targets and bind to the target sequence. The Cas9-endonulcease binds to the sgRNA and cuts the target sequence. By delivering the Cas9 protein and the specific sgRNA targeting a gene of interest into a cell, the host genome can be cut at any targeted location. There are no examples of the successful use of the CRISPR/Cas9 system in microalgae. However, Jiang et al. reported the successful expression of both the Cas9-endonuclease and the sgRNA in *C. reinhardtii*, but with very low examples of successful gene editing events (Jiang et al., 2014). Jiang et al. suggest that the Cas9 is toxic to *C. reinhardtii* and therefore a method needs to be developed that excludes the accumulation of active Cas9 enzymes in transformed cells, e.g. using inducible promoters.

An additional advantage of the CRISP/Cas9 system, is the possibility to obtain marker free genome edited strains. This could be done by the direct delivery of the CRISPR/Cas9 components in the host cell by electroporation. These components, including codon optimized Cas9 mRNA and specific sgRNA targeting the gene of interest, can be synthesized or PCR amplified (DiCarlo et al., 2013; Kaneko et al., 2014; Qin et al., 2015; Ran et al., 2013). This enables the use of the CRISPR/Cas9 system even if an efficient expression system does not exist in the target strains, which is often the case for microalgae. Although, electroporation must work and the codon optimized Cas9 mRNA should be successfully translated into a functional Cas9 protein in the target species to enable this strategy.

This promising technology will need more research to enable more robust and efficient gene editing in microalgae using CRISPR/Cas9. However, since the number of species that has been successfully transformed using CRISPR/Cas9 is increasing every day, it only seems a matter of time before it can be applied in metabolic engineering of microalgae (Belhaj et al., 2015; Jacobs et al., 2014; Jiang et al., 2013).

8.5.1.2 Target gene products to specific subcellular locations

Microalgae contain several different compartments in the cell. The cytoplasm, containing the nucleus, the chloroplast, possibly containing a pyrenoid and the mitochondria. Specific targeting strategies are required in case the product of interest needs to be expressed in a specific compartment. Lately there have been developments in targeting these different compartments, using specific targeting sequences that can be attached to transgenes (Lauersen et al., 2015; Rasala et al., 2014).

8.5.1.3 Mitigating technology

The fitness of transgenic microalgae should be reduced in order to decrease the chance that a transgenic microalgae can survive or even overtake a natural ecosystem. In most cases, the transgenes are highly overexpressed, which is a metabolic burden for the GMOs, reducing their fitness compared to wild type strains without further mitigation being necessary. But on top of this there are some examples to reduce the fitness of transgenic microalgae further, such as reducing the efficiency of the photosystems of the transgenic strain, or reducing the energy capturing capacity. Another possibility is to reduce the carbon concentrating mechanisms. Strains will then need elevated CO_2 concentrations in the cultivation systems to be able to capture enough carbon for growth, although this is economically unfavorable for the production of bulk chemicals. Should these transgenic strains be spilled into natural ecosystems, they will have a reduced fitness. This strategy is referred to as transgenic mitigation, and some other examples include specific nutrient dependence, or removal of flagella in phototactic strains. These features will reduce the fitness and robustness of the production organism in the photobioreactors (PBRs) as well and therefore the need for axenic cultivation is increased.

Ideally a production strain is created that has an increased fitness inside the PBR (for instance high temperature) and decreased fitness outside the PBR (reduced growth at ambient temperatures).

8.5.1.4 Marker free transgenic microalgae

Marker free transgenic strains are preferred over transgenic strains that do contain markers, such as antibiotic resistance genes, to reduce the risk of antibiotic resistance spreading to other organisms. Markers need to be removed from the transgenic strains that are used in large scale production processes. There are several ways to obtain these marker free strains. One of them is by using previously generated auxotrophic mutants (Dawson et al., 1997; Kindle et al., 1989), which need for example uracil, arginine, or ammonium to survive. By combining a gene of interest with the endogenous gene that relieves the auxotrophy (e.g. orotidine 5'-phosphate decarboxylase gene, argininosuccinate lyase and nitrate reductase), the prototrophic growth is restored. Another approach, that is used a lot in selection of marker free GMOs, is to remove the selection marker after successful selection of the transgenic target organism using the Cre-*lox* system. This method can be used to excise a selection marker that is flanked by two *lox* sites from the genome of the transgenic host by the expression of Cre recombinase. This method is proven in many organisms, including yeast, plants, and microalgae (Gilbertson, 2003; Güldener et al., 1996; Heitzer and Zschoernig, 2007).

8.5.2 Lipid productivity

A strong focus in microalgal metabolic engineering should be on improving the lipid productivity. As described in the **chapters 3-5**, blocking the starch biosynthesis will enhance carbon partitioning towards TAG accumulation and increase the lipid productivity of oleaginous microalgae strongly. To enhance the lipid productivity even more, more energy and carbon should be directed towards TAG synthesis. This can be achieved in several ways: increase the photosynthetic efficiency, overexpressing rate limiting genes in the production of TAG, reducing the production of other carbon utilizing pathways, or TAG catabolism. In the transcriptomic study, described in **chapter 2**, we also looked at the genes involved in TAG accumulation in *Neochloris oleoabundans*. It turned out that there is still a lot unclear in the TAG biosynthesis pathways of *N. oleoabundans*, and additional research is needed on the genes involved. Potential target genes for metabolic engineering are the DGAT, ACCase, and the glycerol 3-phosphate acyltransferase and glycerol-3-phosphate dehydrogenase enzymes, which link the glycolysis to the TAG biosynthesis and provide the glycerol backbone for the TAG molecule. The overexpression of a yeast glycerol 3-phosphate acyltransferase in Arabidopsis resulted in a 40% increase in seed oil content (Vigeolas et al., 2007). Overexpression of the endogenous glycerol kinase gene in the oleaginous marine diatom, *Fistulifera solaris*, increased glycerol metabolism and improved the lipid productivity with 12% (Muto et al., 2015).

8.5.2.1 Increase photosynthetic efficiency, especially under nitrogen depletion
The loss in photosynthetic efficiency under nitrogen depleted conditions will reduce the amount of energy and carbon that can be accumulated in TAG, resulting in reduced TAG productivity over time (Klok et al., 2013a). Preventing the decrease in photosynthetic efficiency could result in higher TAG productivity, since there will be more energy available in the form of ATP and NADPH to be stored in TAG (Klok et al., 2014).

8.5.2.2 Simultaneous growth and lipid production
In the current lipid production process, biomass is cultivated and subsequently adverse growth conditions are created, such as nitrogen depletion. This disables the microalgae's ability to channel the photosynthetic energy into biomass, and therefore this energy is channeled towards storage compounds such as TAG. It is hypothesized that the redox/energy imbalance is important for triggering lipid production, although the exact regulatory mechanisms are unknown. Decoupling the TAG accumulation from nitrogen starvation would enable the simultaneous production of lipids and growth at high photosynthetic efficiencies. Klok et al. proved that this concept is possible, however the TAG productivities need to be improved further (Klok et al., 2013a).

Levitan et al. knocked down the nitrate reductase gene in *Phaeodactylum tricornutum*, to 40% of the wild type strain under nitrogen replete conditions. This resulted in a 43% increase in the amount of lipids, under nitrogen replete conditions, without compromising photosynthetic efficiency, however the growth rate of the knockdown transformant were only 70% of the wild type. They suggest that a negative feedback sensor couples photosynthetic carbon fixation to lipid biosynthesis, which is regulated by the nitrogen assimilation pathway. This metabolic feedback enables diatoms to rapidly respond to fluctuations in environmental nitrogen availability (Levitan et al., 2015).

Another way to improve the lipid productivity in microalgae would be to knockdown TAG catabolism. In Arabidopsis, the fatty acid catabolism was knocked out, and resulted in increased TAG contents in leaf tissues (Slocombe et al., 2009). In microalgae, a similar approach was used to increase the lipid productivity in the diatom *Thalassiosira pseudonana*, by knocking down a multifunctional lipase/phospholipase/acyltransferase using RNA silencing (Trentacoste et al., 2013). The knockdown strains showed increased lipid content during exponential growth and silicon starvation. The authors suggest a role for the knocked down lipase in membrane lipid turnover and lipid homeostasis.

These results demonstrate the potential that targeted metabolic engineering approaches have, which can be used to increase lipid accumulation in growing eukaryotic microalgae. Another advantage of blocking TAG catabolism would also be the reduction of nocturnal TAG losses due to respiration.

8.5.3 Cost reduction

Another important strategy to make exploitation of microalgal lipids a step closer to reality is by reducing the production costs. As an additional benefit, most of the cost reducing measurements are caused by reduction of resources or energy input, which also impact the sustainability of the process positively. For example the use of salt water instead of fresh water in large scale production processes. The use of salt water is more sustainable and cost effective compared to fresh water, and will reduce the fresh water footprint of the production process. Salt water is abundantly available for a lower price compared to freshwater.

8.5.3.1 Increase salt tolerance

A strong asset of using microalgae to produce products, such as oil, over traditional plant crops, is the possibility to use salt water, hereby reducing the fresh water footprint of the process. However, not all microalgae are able to grow in salt water conditions. In **chapter 2** the salt resistance mechanism of the freshwater oleaginous microalgae *N. oleoabundans* was studied. Sequencing of the mRNA of this species under fresh water and salt water conditions revealed that *N. oleoabundans* is accumulating proline to protect itself from osmotic stress. In addition to proline accumulation, there is an increase in the expression of genes involved in removing reactive oxygen species using the glutathione-ascorbate cycle. This indicates that the salt stress is resulting in higher oxidative stress. Overexpressing rate limiting genes in proline biosynthesis and the glutathione-ascorbate cycle, might increase the salt tolerance of other microalgal species as well.

8.5.3.2 Product secretion

One of the most expensive steps in the production of microalgal products is the extraction and purification of the product from the microalgal biomass. One possible solution would be to metabolically engineer microalgae to secrete the product of interest directly into the growth medium. The process of protein secretion by microalgae is well understood, and there are several examples of successful production and secretion of recombinant proteins in *C. reinhardtii* (Lauersen et al., 2013; Rasala et al., 2012). Lipid secretion is not naturally occurring in microalgae. However, there are examples of yeast mutants that can secrete free fatty acids or even TAG molecules, as a result of the inactivation of genes involved in β-oxidation, such as acyl-CoA oxidase and several acyl-CoA synthetases (Nojima et al., 1999; Scharnewski et al., 2008). In Cyanobacteria, the introduction of acyl-acyl carrier protein thioesterases resulted in fatty acid excretion as well (Liu et al., 2010).

Another promising way to make microalgae secrete lipids would be by the introduction of ABC transporters. These transporters can export waxes, long chain fatty acids, and

carbohydrates over the membrane of plants. Although the exact mechanism is not yet well understood, there are examples of transgenic plants that can secrete such molecules using the ABC transporters (Mentewab and Stewart, 2005; Yu et al., 2005).

A challenge that arises when energy dense molecules such as free fatty acids or TAG are secreted into the cultivation medium is the increased need for axenic cultivation, due to potential growth of contaminants.

8.5.3.3 Inducible auto-flocculation

Auto-flocculation would be a promising tool to reduce the costs in downstream processing, since less energy is needed to separate the biomass from the culture medium (Salim et al., 2011). An inducible auto-flocculation mechanism can be engineered in microalgae, which can be activated at the end of the production process, prior to harvesting. The microalgal cells will produce cationic polymers which are excreted and presented on the outside of the cell wall. The positively charged polymers bind to microalgal cells, which causes the formation of microalgal aggregates that will settle very quickly.

8.5.3.4 Nitrogen fixation

A very complex and potentially profitable strategy would be to let microalgae fix their own nitrogen. This would reduce the dependence on fertilizers, which are mostly derived from petrochemical processes and are a costly part of the cultivation process. There are cyanobacteria known that are able to fix nitrogen, while almost all eukaryotic microalgae species require an exogenous source of fixed nitrogen, such as ammonium or nitrate. The most imaginative nitrogen fixing prokaryotic microalgae is *Anabaena*. This filamentous cyanobacteria is known for its nitrogen fixing abilities in the heterocysts, which are distributed along the filaments. The heterocysts maintain a microoxic environment to enable nitrogen fixation at the expense of ATP and reductants, which are provided by the photosynthetic vegetative cells (Kaneko et al., 2001). The co-cultivation of nitrogen fixing bacteria and lipid producing microalgae would be interesting to investigate. In fact, there are two diatoms, *Rhopalodia gibba and Climacodium frauenfeldianum*, that have acquired nitrogen fixing cyanobacteria by endosymbiosis (Carpenter and Janson, 2000; Kneip et al., 2008). This is a very interesting starting point to explore the potential of nitrogen fixation by eukaryotic microalgae.

8.5.4 Robustness

8.5.4.1 Microalgal-microbe communities

Maintaining stable axenic microalgal populations in large scale production systems is challenging. Using a community of different microalgal species or a consortium of microalgae and other microbes can increase the stability of the culture, or even the produc-

tivity of the entire population (Kazamia et al., 2012a). The concept of synthetic ecology may not require metabolic engineering, but molecular biology tools are needed to study the dynamics and mutual interactions between the different species. In some cases bacteria might supplement the microalgal cells with essential nutrients, such as vitamins (Kazamia et al., 2012b).

8.5.4.2 High temperature tolerance

In outdoor cultivation systems temperatures may vary a lot during the day and night. The irradiation of the sun will heat up the inside of the photobioreactors severely. Unless active cooling is applied, cells will be damaged by these temperatures. Increasing the heat tolerance of industrial strains would reduce the need for cooling and make the strains more robust. However, temperature tolerance is difficult to improve, since this requires the modification of many enzyme that are involved in the metabolism. In addition to optimizing the heat tolerance of the enzymes, the lipid composition of the membranes is influenced by temperature and would need improvement as well (James et al., 2013). The use of laboratory evolution might be a more feasible approach to increase the temperature tolerance.

8.6 How to get genetically engineered microalgae on the market

Genetic engineering of microalgae is needed to make microalgal derived products competitive with currently existing microbial cell factories and crop plants. Currently, obtaining GM microalgae might be the biggest challenge but implementing them on the market will be the next challenge and needs to be addressed as well. There are several issues concerning the authorization of GM microalgae. The legislation for production and application in consumer products is discussed, as well as consumer perception with regard to GMOs.

8.6.1 Legislation for production

With the emergence of biotechnology and genetic engineering of organisms in the 1970s and 1980s, the European Union needed to develop policy to regulate the production and distribution of GMOs. The regulatory system is based on three pillars. First, the safety of humans, animals, and the environment must be secured. Second, the legislation should be equal and harmonized throughout Europe. Third, consumers should be able to make a free, well informed, choice for GMO containing products. To enforce these three basic rules the EU has developed the following regulatory framework. There is a clear distinction between contained use of GMOs and deliberate release of GMOs into the environment. Contained use of GMOs is covered in the EC Directives 2009/41/EC and the deliberate release of GMOs into the environment is covered by EC Directive 2001/18/EC (See box 8.1 for definitions).

In both directives the performance of an environmental risk assessment (ERA) is obligatory. In Directive 2001/18/EC an ERA is defined as "*the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMOs may pose*". Under Directive 2009/41/EC "human health" is taken into consideration only as far as incidental exposure is concerned, since the GMOs will be contained. When products for food and feed applications contain or are composed of GMOs, Regulation 1829/2003 (EU, 2003) applies.

Box 8.1

Contained use:
"*any activity in which (micro-)organisms are genetically modified or in which such (micro-)organisms are cultured, stored, transported, destroyed, disposed of or used in any other way and for which specific containment and other protective measures are used to limit their contact with the general public and the environment*" (EU, 2009)

"*contained use, means any activity in which micro-organisms are genetically modified (…) and for which specific containment measures are used to limit their contact with the general population and the environment*" ((EU, 2009), article 2c)

Deliberate release in the environment:
"*any intentional introduction into the environment of a GMO or a combination of GMOs for which no specific containment measures are used to limit their contact with, and to provide a high level of safety for, the general population and the environment*". (EU, 2001)

Good Industrial Large Scale Practice
When there is a history of safe and Good Industrial Large Scale Practice (GILSP) with the wild type strain, genetically modified strains can be regarded under the GILSP legislation (OECD, 1986). This applies only when the host is non-pathogenic and long history of safe use in industrial settings and the GMO contains a "safe" insert and vector, and a lower fitness in the environment compared to the wild type. The GILSP concept will not be the main rule that applies for microalgae in the near future, since there is relatively limited history of large scale microalgal cultivation in an industrial setting. Currently there is therefore no approval for outdoor large scale production of GM microalgae in the Netherlands and only a few examples of production facilities for non-GMO microalgae are known. These current production facilities can be used in the future to serve as references for GILSP legislation.

Microalgae do not necessarily fit into these definitions, because the regulations are designed for industrial microorganism that are cultivated in stainless steel tanks in factories, or designed for GM crops, which are cultivated outdoors on agricultural land. Three

commonly used production systems for large-scale microalgal cultivation can be distinguished. The most basic approach would be cultivation of microalgae in large natural locations which is done, for example, worldwide with *Dunaliella* strains. Releases in natural locations are evidently regarded as deliberate release into the environment, since there are no effective protective measurements to prevent the algae from entering the surrounding environment. The most widely used production system are open raceway ponds (Figure 8.2A), which are large open structures. When these raceway ponds are used outdoors, the cultivation will be regarded as deliberate release. The ponds are not covered, so spread of the GMO cannot be excluded. However, when the ponds are placed in greenhouses with precautions to prevent any exchange of biomaterial with the outside world, one might be able to get the contained cultivation label. Thirdly there are the more sophisticated, closed production systems, photobioreactors (PBR, figure 8.2B-C). These structures can be placed in greenhouses (Figure 8.2C) to have optimal containment and therefore the existing regulation is applicable (COGEM, 2012). The question is whether closed PBRs, which are placed outdoors, can be considered as contained cultivation when they comply with the EU regulations described in box 8.1. This has not yet been done, but according to the current regulations, there should be room for approval (COGEM, 2012).

Figure 8.2 Different systems used in microalgal cultivation. (A) Open raceway ponds. (B) Photobioreactors. (C) Photobioreactor inside a greenhouse.

8.6.2 Environmental risk assessment

Part of the approval for production of GMOs is an assessment to evaluate the impact of the GMO on the natural ecosystem. The safety of a strain should be investigated thoroughly, involving pre-existing data on mass culture and also the possibility that the organism produces harmful compounds, such as toxins (IOC-UNESCO list of harmful algae). In general, green microalgae are harmless to human health and the environment. There are some microalgae that received the Generally Regarded As Safe (GRAS) label to be produced for feed and food (such as *C. reinhardtii*, *Dunaliella sp.*, and *Haematococcus pluvialis*), while some species are allowed based on the history of consumption (such as *Chlorella vulgaris*). The fitness of the GM microalgae with respect to the wild type needs to be addressed and preferably the GM microalgae should have a lower fitness than the wild type (see examples of mitigation have been discussed earlier).

The risk of possible mingling of recombinant DNA into other species in the environment needs to be addressed. This could happen by sexual reproduction with very closely related species, or by horizontal gene transfer (HGT). HGT is the transfer of genetic material between organisms, other than traditional reproduction. This is of concern because it could result in the exchange of transgenes encoding antibiotic resistance or competitive advantages in the wild type donor species. HGT has been part of the evolutionary development in microalgae, for example the prokaryotic origin of plastids that occurred millions of years ago (Derelle et al., 2006; Keeling and Palmer, 2008; Qiu et al., 2013; Rogers et al., 2007). HGT in microalgae should be studied in more detail, therefore the risk that genetic material from microalgae is transferred to other organisms in the environment cannot be excluded. To reduce the risk of HGT of antibiotic resistance genes from GMOs to wild type organisms, the use of marker free GMOs is recommended. These GMOs do not contain an antibiotic resistance marker and the chances of getting such a GMO approved are larger than with strains containing antibiotic resistance markers. In case the GMO is to be deliberately released in the natural environment, antibiotic markers are not allowed. Strategies for generating marker free strains were discussed earlier in this chapter.

8.6.3 Legislation to get GMO derived products in consumer products

All novel foods and novel food ingredients that are introduced need to be legislated case by case, under the Regulation 258/97/EC (EU, 1997). New microalgal (products) are authorized every now and then, e.g. the use of DHA and EPA-rich oil from the microalgae *Schizochytrium sp.* as a novel food ingredient (Decision 2015/546). In the past, the substantial equivalence principle was applied (Regulation 258/97/EC) for products made from GMOs in which the end product is recombinant DNA or protein free. In the US this is still covered in the conventional regulations on novel foods and food ingredients and no special legislation is needed. In the EU, the Regulation 1829/2003/EC is in effect when products contain or are composed of GMOs. Accidental GMO contamination of up to 0.9% with an authorized GMO in the food or feed product is exempted from the regulation. When the product is derived from a non-authorized GMO, the residues should be below the detection limit, 0.0%. For approval under this regulation, appropriate labeling is required (Regulation 1830/2003), as well as a safety assessment according to the European Food and Safety Authorization (EFSA) guidelines.

The legislation in the USA is based on the belief that biotechnological intervention resulting in GMOs does not introduce risks by itself (Echols, 1998). This basic principle enables the use of existing regulatory frameworks and institutions. GM products are assessed in the same framework as conventional non-GMO products, by the Environmental Protection Agency (EPA) United States Department of Agriculture (USDA) and Food and Drug Authority (FDA). To get approval in the EU takes much more time than in the US,

since the authorization process is done on a very high political level involving all member states (Davison, 2010). This accounts especially for GMOs that will be intended for release in the environment, as GM microorganisms, cultivated in containment, can be approved on a member state level and countries are advised by their National Competent Authority. The scientific fact based advice from the EFSA is the foundation of the decision to authorize GMOs by the ministers of all member states, but also political and societal factors play a role (Davison, 2010). In the US the approval is done on a federal level by regulating agencies and not by the individual states. Therefore, the process is technocratic instead of political (Sheingate, 2009).

8.6.4 Consumer perception

To introduce new technologies on the market, consumer acceptance is very important. And the discussion with consumers, policy makers, scientists and environmental non-governmental organizations (NGO) should be facilitated. In the meantime, it is important that research is being done on generating GM microalgae, to progress the research on specific targets for strain improvement as described earlier. The outcome of this research could be used to formulate the advantages, disadvantages, and risks associated with GM microalgae. Also, the knowledge that is gained from metabolic engineering of microalgae can be used to develop non-GM microalgae with similar traits using reverse engineering.

The public opinion on GMOs is best monitored using large surveys, which show that the consumer acceptance and trust in modern biotechnology is very different between countries and cultures (Bonny, 2008; Hallman et al., 2004). The percentage of people that think biotechnology will improve their standard of living in the next 20 years, is the highest in Scandinavian countries. Sweden tops the list at 80% of the people who believe biotechnology will improve their way of living. On average, 56% of the Europeans is agreeing with this proposition, compared to 72% of the people in the US (Bonny, 2008).

8.7 Conclusion

Today, bulk products from microalgae are not yet economically feasible, but for many higher value biomolecules there is much potential on the short term. Genetic engineering needs to be applied in order to reduce the overall production costs of microalgae derived products to such an extent that they become economically viable. One of the main challenges in metabolic engineering of microalgae are the rigid cell walls, such as algaenan containing cell walls. We have developed a method to quantify the efficiency of the selected transformation parameters, and a method is proposed to select for mutants with a reduced cell wall rigidity that are more accessible to metabolic engineering. As long as the genetic engineering toolboxes are not available for microalgae, strain

improvement of microalgae will rely on classic strategies such as mutagenesis, laboratory evolution and selective breeding techniques, for which examples are provided in this thesis. Once GM microalgae with enhanced properties are created, they need to be authorized by the regulatory institutions. The most likely case for GM microalgae to be accepted in Europe would be comply to the following features: GRAS status species, grown in contained PBR in a greenhouse. The transgene that is included enhances productivity of a not harmful compound, does not contain antibiotic resistance markers, and is accompanied by a fitness reducing genetic system.

A public discussion on the legislation of GM microalgae is suggested, together with the submittal of applications for a permit to cultivate GM microalgae on pilot scale at scientific institutions or universities. This will be the first step enabling answers to questions regarding, bio-safety, stability and fitness compared to wild type strains. Finally, researchers should face the public debate and explain in a clear and general objective way the advantages and disadvantages of GMOs.

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Summary

The increasing world population and living standards have enlarged the demand for food, feed, and for chemicals. Traditional fossil fuel based commodities need to be replaced, not only because these resources are finite, but also to relieve the impact of carbon emission and pollution, resulting from fossil fuel derived processes. Much attention is on using plants to produce sustainable, renewable alternatives to petrochemical based processes. Palm oil is the crop with the highest lipid yield known today, but the production of palm oil causes deforestation on a large scale. In the past decades, researchers have been looking for an alternative agricultural crop that has a high productivity and does not have such a large impact on available cropland.

Microalgae are a promising platform for the production of sustainable commodity products, such as pigments, proteins, carbohydrates and lipids. Microalgae use the energy of the sun to convert carbon dioxide into sugars, which are the building blocks for many molecules, such as lipids and starch. They can be grown year round on non-arable land, using sea or waste water and a few added nutrients. A commodity product that can be produced in microalgae is triacylglycerol (TAG), a glycerol molecule with 3 fatty acyl chains attached. The TAG molecules that are accumulated in microalgae are comparable to the TAG profiles of commonly used vegetable oils, and can directly be applied for edible oil as well as for biodiesel production. Currently, microalgae derived products have proven to be functional and a potential replacement for conventional crops. However, microalgae derived products, especially TAGs, are not economically feasible yet. In order to make microalgal derived products a reality we need to decrease the production costs by smart technological solutions, biological understanding and metabolic engineering. The aim of the work described in this thesis was to explore the lipid production mechanisms in oleaginous microalgae and to apply this knowledge to improve microalgal lipid productivity and lipid composition using strain improvement strategies.

To get more insight in the lipid accumulation mechanism of microalgae, and to define targets for future strain improvement strategies, transcriptome sequencing of the oleaginous microalgae *Neochloris oleoabundans* was done. This oleaginous microalga can be cultivated in fresh water as well as salt water. The possibility to use salt water gives opportunities for reducing production costs and fresh water footprint for large scale cultivation. In **chapter 2** *N. oleoabundans* was cultivated in photobioreactors under four different conditions: fresh water nitrogen replete and depleted conditions, and salt water nitrogen replete and depleted conditions. The lipid accumulation pathway was studied to gain insight in the gene regulation 24 hours after nitrogen was depleted. Oil accumulation is increased under nitrogen depleted conditions in a comparable way in both fresh and salt water. The transcriptome sequencing revealed a number of genes, such as glycerol-3-phosphate acyltransferase and via glycerol-3-phosphate dehydrogenase, that are of special interest and can be targeted to increase TAG accumulation in microalgae.

The salt resistance mechanism is studied in detail using NMR spectroscopy in addition to transcriptome analysis. NMR spectroscopy revealed an increase in proline content in saline adapted cells, which was supported by up regulation of the genes involved in proline biosynthesis. In addition to proline, the ascorbate-glutathione cycle seems to be of importance for successful osmoregulation by removal of reactive oxygen species in *N. oleoabundans*, because multiple genes in this pathway were upregulated under salt conditions. The mechanism behind the biosynthesis of compatible osmolytes in *N. oleoabundans* can be used to improve salt resistance in other industrially relevant microalgal strains.

Another very promising candidate for TAG production is the oleaginous green microalga *Scenedesmus obliquus*, because of its high lipid productivity and capability of maintaining its photosynthetic efficiency upon nitrogen starvation. Under these conditions, starch is accumulated as well and can reach up to 38% (% cell dry weight, CDW), which is a substantial part of the total carbon captured. In **chapter 3**, UV mutagenesis was used to create starchless mutants, since no transformation approach was available for this species, due to its rigid and robust cell wall. Starchless mutants of *S. obliquus* were isolated by screening for reduced starch content using Iodine vapor staining. All five starchless mutants that were isolated from over 3500 screened mutants, showed an increased triacylglycerol productivity. The effect of the mutation on biomass and total fatty acid (TFA) and TAG productivity under nitrogen replete and nitrogen-depleted conditions was studied. All five starchless mutants showed a decreased or completely absent starch content. In parallel, an increased TAG accumulation rate was observed for the starchless mutants and no substantial decrease in biomass productivity was perceived. The most promising mutant (Slm1) showed an increase in TFA productivity of 41% at 4 days after nitrogen depletion and reached a TAG content of 49.4% (%CDW).

In **chapter 4** the Slm1 strain was compared to the wild type strain. The use of photobioreactors enabled frequent biomass sampling over time to study the differences in biomass composition between the two strains under defined light conditions. In the wild type, TAG and starch accumulated simultaneously during initial nitrogen starvation, and starch was subsequently degraded and likely converted into TAG. The Slm1 did not produce starch and the carbon and energy acquired from photosynthesis was partitioned towards TAG synthesis. This resulted in an increase of the maximum TAG content in Slm1 to 57% (%CDW) compared to 45% (%CDW) in the wild type. Furthermore, it increased the maximum yield of TAG on light by 51%, from 0.144 in the wild type to 0.217 g TAG mol^{-1} $photon^{-1}$ in the Slm1 mutant. No differences in photosynthetic efficiency between the Slm1 mutant and the wild type were observed, indicating that the mutation specifically improved carbon partitioning towards TAG and the photosynthetic capacity was not affected.

To identify the mutation that caused the starchless phenotype of Slm1 the transcriptome of both the wild type and the Slm1 mutant was sequenced as described in **chapter 5** and the expression of the starch biosynthesis genes under nitrogen replete conditions was studied. UV mutagenesis introduces transversions in the genome resulting in a point mutation in the genome. A single nucleotide polymorphism (SNP) was discovered in the small subunit of the starch biosynthesis rate-controlling enzyme ADP-glucose pyrophosphorylase, which resulted in the introduction of a STOP codon in the messenger RNA of the enzyme. The mutation was confirmed by sequencing the PCR product of the small subunit of the ADP-glucose pyrophosphorylase gene in the wild type and Slm1 strains.

The characterization of the mutation increases the understanding of carbon partitioning in oleaginous microalgae, leading to a promising target for future genetic engineering approaches to increase TAG accumulation in microalgae.

To use the insight that is gained in **chapters 2-5** for metabolic engineering of TAG accumulation and carbon partitioning, a metabolic engineering toolbox is required. However, the development of transformation protocols for new and less well studied industrially relevant microalgae is challenging. The unexplored physiology and largely unknown genomes are a bottleneck in the successful transformation of these species. This leads to the use of suboptimal genetic constructs, based on standardized vectors for plant and microalgal model species. However, one of the main reasons for unsuccessful transformation of microalgae is the poor intracellular delivery of exogenous DNA.

In **chapter 6**, a simple and effective tool for the optimization of transformation protocols is proposed. For successful transformation of microalgae, we need insight in the parameters that result in permeabilization of the cells to enable the uptake of exogenous DNA. However, when cells are permeabilized too much, the cells will rupture and will not survive the procedure. Thus, there is a delicate balance between successful transformation and cell death, which is different for each species depending on the cell wall composition. Cell competence can be monitored by measuring cell permeabilization and viability separately using Sytox Green and Propidium Iodide respectively. Optimal voltage settings were determined for five microalgae: *C. reinhardtii*, *Chlorella vulgaris*, *N. oleoabundans*, *S. obliquus*, and *Nannochloropsis sp.* The results confirm the previous observation that the oleaginous species *S. obliquus*, *N. oleoabundans* and *Nannochloropsis sp.* have a more robust cell wall and that more harsh conditions are needed to make these cells permeable. The optimal voltage settings obtained for the model strains *C. reinhardtii* and *C. vulgaris* are in correspondence with the values found in literature. This method can be used to speed up the screening process for species that are susceptible for transformation and to successfully develop transformation strategies for industrially relevant microalgae, which lack an efficient transformation protocol.

Increasing the TAG productivity of microalgae is crucial in obtaining an economically feasible production platform. In addition to the increase in productivity, improving the quality in terms of fatty acid composition of TAG molecules would be desired as well. In case we can design and develop tailor made TAG molecules with a specific fatty acid profile, the applications of microalgal TAG can be extended. For example, the accumulation of stearic acid rich TAG molecules are of special interest, because of the improved structural properties. However, since the toolbox for transforming oleaginous microalgae is not yet well established, we used a lipid accumulating starchless mutant of the model species *C. reinhardtii* BAFJ5 as our host in **chapter 7** as a proof of concept. In this chapter, stearoyl-ACP desaturase (SAD), the enzyme that converts stearic acid into oleic acid, is silenced by artificial microRNA. Two different constructs, which target different positions on the mRNA of stearoyl-ACP desaturase, were tested. For each construct, three transformants were cultivated and the total fatty acid and triacylglycerol content was measured at two temperatures over time. The mRNA levels for SAD were reduced after the silencing construct was induced. In one of the strains, the reduction in SAD mRNA resulted in a doubling of the stearic acid content in triacylglycerol molecules, which shows that increasing the fraction of stearic acid in TAG is possible. Furthermore, we hypothesize that in addition to direct conversion in the chloroplast, *C. reinhardtii* is able to redirect stearic acid from the chloroplast to the cytosol and convert it to oleic acid in the endoplasmic reticulum by stearoyl-CoA desaturase.

In **chapter 8**, an outlook is given on microalgal strain improvement strategies for the future, reflecting on the results obtained in this thesis. Also a roadmap is suggested to get genetically modified microalgal derived products on the market. In the near future, microalgal metabolic engineering will rely on classic strategies such as mutagenesis, laboratory evolution and selective breeding techniques, for which examples are provided in this thesis. A selection method is proposed to select for mutants with a reduced cell wall rigidity, which are more accessible to metabolic engineering. Once GM microalgae with enhanced properties are created, they need to be authorized by the regulatory institutions. The most likely case for GM microalgae to be accepted in Europe would comply with the following features: GRAS status species, grown in contained PBR in a green house. The transgene that is included enhances productivity of a not harmful compound, does not contain antibiotic resistance markers, and is accompanied by a fitness reducing genetic system.

The results presented in this thesis, provide a significant improvement in the understanding of TAG accumulation and carbon partitioning in oleaginous microalgae. Furthermore, improved microalgal strains with increased TAG accumulation or improved TAG fatty acid composition under nitrogen depleted conditions were generated. In addition, an outlook is presented in which the major bottlenecks are presented in future industrial

applications of microalgae. And I hope that with the work described in this thesis, sustainable microalgal derived (vegetable) oils will be one step closer to reality.

Samenvatting

De groeiende wereldbevolking en toenemende levensstandaard van opkomende economieën zorgen voor een oplopende behoefte aan voedsel, (vee)voer en chemicaliën. Traditionele grondstoffen die worden gewonnen uit fossiele bronnen dienen te worden vervangen, niet alleen omdat deze bronnen eindig zijn, maar ook om de uitstoot van koolstofdioxide en vervuiling te verminderen. Veel aandacht gaat uit naar het gebruik van planten als duurzaam hernieuwbaar alternatief voor de huidige petrochemische processen. Palmolie is het gewas met de hoogste olie productiviteit, maar de productie van palmolie resulteert in grootschalige ontbossing van tropisch regenwoud in landen als Indonesië en Maleisië. De laatste jaren zijn wetenschappers druk bezig geweest met het vinden van alternatieve agrarische productiegewassen met een hoge productiviteit die weinig impact hebben op de beschikbare vruchtbare landbouwgrond.

Microalgen zijn een veelbelovend platform voor de productie van duurzame grondstoffen, zoals pigmenten, eiwitten, koolhydraten en oliën. Microalgen kunnen de energie van zonlicht gebruiken om koolstofdioxide om te zetten in suikers, welke de bouwstenen zijn voor vele bruikbare moleculen zoals zetmeel en olie. Ze kunnen het hele jaar door gekweekt worden op land dat niet geschikt is voor de landbouw, met behulp van zee- of afvalwater en toegevoegde nutriënten. Een van de grondstoffen die kan worden geproduceerd met behulp van microalgen is triacylglycerol (TAG), een glycerol molecuul met drie vetzuur staarten. De TAG moleculen die algen produceren zijn vergelijkbaar van samenstelling als de TAG moleculen van veelgebruikte plantaardige oliën en kunnen ook worden gebruikt voor biodiesel productie. Het inpassen van olie uit microalgen is nu al technisch haalbaar, economisch gezien kan het nog niet uit om microalgen olie toe te passen als bulk grondstof. Om de toepassing van producten uit microalgen te realiseren zullen de productie kosten omlaag moeten door technologische ontwikkelingen, meer inzicht in de biologie van de microalgen en genetische modificatie. Het doel van het werk beschreven in dit proefschrift is om het olieproductiemechanisme in microalgen te onderzoeken en om deze kennis te gebruiken om de samenstelling van de olie te verbeteren en de olie productiviteit te verhogen door middel van stamverbetering technieken.

Om meer inzicht te krijgen in de olie productie mechanismen van microalgen, en om toekomstige doelen te formuleren voor stamverbetering, is het transcriptoom van de olieproducerende microalg *Neochloris oleoabundans* gesequensed. Deze olieproducerende microalg kan worden gekweekt in zowel zoet- als zoutwater en dit biedt mogelijkheden om de productiekosten en de zoetwater voetafdruk te verlagen als microalgen gekweekt worden op grote schaal. In **hoofdstuk 2** staat beschreven hoe *N. oleoabundans* gekweekt werd in fotobioreactoren onder vier verschillende condities: zoetwater met en zonder stikstof en zoutwater met en zonder stikstof. De olie productie route werd

bestudeerd om inzicht te krijgen in de gen regulatie 24 uur nadat de stikstof was verwijderd uit het medium. Olie werd op een vergelijkbare manier opgehoopt in zowel de zoet- als de zoutwater aangepaste cellen. Het sequensen van het transcriptoom bracht een aantal genen naar voren, zoals glycerol-3-fosfaat acyltransferase en glycerol-3-fosfaat dehydrogenase, die interessant kunnen zijn om de TAG ophoping in olierijke microalgen nog meer te verhogen. Het zout resistentie mechanisme is, naast het sequensen van het transcriptoom, in meer detail bestudeerd met behulp van NMR spectroscopie. NMR spectroscopie liet zien dat er een toename is in concentratie van het vrije aminozuur proline in cellen die groeien in zoutwater. Bovendien komen de genen die zijn betrokken in de proline biosynthese meer tot expressie in zoutwater geadapteerde cellen. Naast proline is ook de ascorbaat-glutathion cyclus van belang om in zoutwater geadapteerde cellen de osmoregulatie op peil te houden door het verwijderen van reactieve zuurstof radicalen in *N. oleoabundans*. De mechanismen achter de biosynthese van compatibele osmolyten in *N. oleoabundans* kunnen worden gebruikt om andere industrieel relevante microalgen resistenter te maken tegen zoutwater.

Een andere veelbelovende kandidaat voor de productie van TAG is de olierijke microalg *Scenedesmus obliquus* door de hoge olie productiviteit en de eigenschap dat het lang haar fotosynthetische efficiency kan handhaven nadat de stikstof op is. Onder deze condities wordt naast TAG ook zetmeel opgehoopt, tot wel 38% van het drooggewicht, wat een substantieel deel is van de totale koolstof die is vastgelegd. In **hoofdstuk 3** wordt beschreven hoe met behulp van UV mutagenese zetmeelarme mutanten zijn gemaakt. UV mutagenese is gebruikt omdat er voor *S. obliquus* nog geen methode is ontwikkeld om deze microalg genetisch te modificeren door de robuuste en stevige celwand van deze microalg.

Zetmeelarme mutanten zijn geïsoleerd door het zetmeel in de mutanten te kleuren met Jodium gas. Vijf zetmeelarme mutanten werden geïsoleerd uit totaal 3500 onderzochte mutanten en alle zetmeelarme mutanten hadden een verhoogde TAG productiviteit. Het effect van de mutatie op de biomassa samenstelling, de totale vetzuur en TAG productiviteit onder stikstof rijke en arme condities werden bestudeerd. Alle vijf de mutanten hadden een verminderde of zelfs complete afwezigheid van zetmeel. Parallel hieraan hadden de zetmeelarme mutanten een verhoogde TAG productie snelheid en was er geen negatief effect waarneembaar op de biomassa productiviteit. De zetmeelarme mutant met de meeste potentie (Slm1) had een toename in de totale vetzuur productiviteit van 41% na vier dagen van stikstof stress en haalde een TAG concentratie van 49.4% (van het drooggewicht).

In **hoofdstuk 4** wordt de Slm1 mutant in meer detail vergeleken met de wildtype stam. Door het gebruik van fotobioreactoren konden meerdere monsters worden genomen

om de verschillen in biomassa samenstelling tussen beide stammen te onderzoeken bij gecontroleerde lichtomstandigheden gedurende het experiment. In het wildtype werden TAG en zetmeel tegelijkertijd opgehoopt in het begin van de stikstof stress fase en later werd het zetmeel afgebroken en mogelijk alsnog omgezet in TAG. De Slm1 stam hoopte geen zetmeel op en de vrijgekomen suikers en energie uit fotosynthese werd volledig gebruikt om TAG op te hopen. Dit resulteerde in een toename van de maximale TAG productiviteit in de Slm1 stam naar 57% (van het drooggewicht) ten opzichte van 45% (van het drooggewicht) in het wildtype. Hierdoor nam de maximale yield van TAG per hoeveelheid licht toe met 51% van 0.144 in het wildtype naar 0.217 g TAG mol^{-1} $photon^{-1}$ in de Slm1 mutant. Er waren geen verschillen waarneembaar in fotosynthetische efficiëntie tussen de Slm1 mutant en het wildtype. Dat geeft aan dat de zetmeelarme mutant specifiek de koolstof allocatie naar TAG verhoogt en dat de fotosynthetische capaciteit niet wordt beïnvloed.

Om de mutatie die het zetmeelarme fenotype veroorzaakt in de Slm1 stam te karakteriseren is het transcriptoom van zowel de Slm1 mutant als het wildtype gesequensed en de resultaten zijn beschreven in **hoofdstuk 5**. De expressie van de zetmeel biosynthese genen is bestudeerd onder optimale groeiomstandigheden. UV mutagenese kan transversies induceren in het genoom, waardoor puntmutaties kunnen optreden. Een enkel-nucleotide-polymorfie (SNP) werd ontdekt in de kleine subunit van het enzym ADP-glucose pyrofosforylase, wat resulteert in de introductie van een STOP codon in het boodschapper-RNA van het enzym. De mutatie is bevestigd door het PCR fragment van de kleine subunit van het ADP-glucose pyrofosforylase, van zowel het wildtype als de Slm1 mutant te sequensen.

De karakterisatie van de mutatie geeft meer inzicht in de koolstof allocatie in olierijke microalgen en is een veelbelovend doelwit voor toekomstige genetische modificatie aanpakken om de TAG ophoping in microalgen te verbeteren.

Om de inzichten die zijn opgedaan in **hoofdstuk 2-5** te kunnen gebruiken om TAG ophoping en koolstof allocatie te verbeteren met behulp van genetische modificatie, moet een genetische modificatie *toolbox* beschikbaar zijn. Echter de ontwikkeling van transformatie protocollen voor minder goed bestudeerde microalgen die relevant zijn voor industriële toepassingen is een uitdaging. De onontgonnen fysiologie en veelal onbekende genomen zijn een knelpunt in de succesvolle transformatie van deze soorten. Dit leidt tot het gebruik van suboptimale genetische constructen op basis van gestandaardiseerde vectoren bedoeld voor planten en model algen. Één van de belangrijkste redenen voor het tekortschieten van transformatie van microalgen is de slechte intracellulaire aanvoer van exogeen DNA.

In **hoofdstuk 6** wordt een eenvoudige en doeltreffende methode voor de optimalisatie van transformatieprotocollen voorgesteld. Om microalgen succesvol te transformeren is inzicht in de verschillende parameters die resulteren in de doorlaatbaarheid van de cellen en daarmee de opname van exogeen DNA mogelijk maken wenselijk. Echter, wanneer de cellen te veel poriën krijgen, kunnen de cellen scheuren en de procedure niet overleven. Er is dus een delicaat evenwicht tussen succesvolle transformatie en celdood, die uniek is voor elke soort, afhankelijk van de samenstelling van de celwand. De competentie van cellen kan worden gemonitord door de cel permeabiliteit en de levensvatbaarheid afzonderlijk te bepalen door gebruik te maken van respectievelijk Sytox Green en propidiumjodide, twee fluorescente kleurstoffen. Optimale voltage instellingen zijn bepaald voor vijf microalgen: *C. reinhardtii*, *Chlorella vulgaris*, *N. oleoabundans*, *S. obliquus* en *Nannochloropsis sp*. De resultaten bevestigen de eerdere constatering dat de oliehoudende soorten *S. obliquus*, *N. oleoabundans* en *Nannochloropsis sp*. een meer robuuste celwand hebben en dat een hoger voltage nodig is om deze cellen permeabel te maken. De optimale voltage instellingen die zijn gevonden voor de model soorten *C. reinhardtii* en *C. vulgaris* zijn in overeenstemming met waardes uit de literatuur. Deze methode kan worden gebruikt om snel soorten die vatbaarder zijn voor transformatie te isoleren en om transformatie strategieën voor industrieel relevante microalgen, welke vaak geen doeltreffende transformatieprotocollen hebben, te ontwikkelen.

Het verhogen van de TAG productiviteit in microalgen is cruciaal om microalgen als productieplatform economisch haalbaar te maken. Naast het verhogen van de productiviteit, kan ook gewerkt worden aan het verhogen van de kwaliteit van de vetzuursamenstelling van de TAG moleculen. Als we in staat zijn om de vetzuursamenstelling van de TAG moleculen op maat te maken met specifieke vetzuurprofielen kunnen we de kwaliteit en daarmee de waarde van de TAG moleculen verhogen. Bijvoorbeeld de accumulatie van TAG moleculen rijk aan stearinezuur zijn interessant, omdat deze moleculen verbeterde structuur eigenschappen hebben. Aangezien de *toolbox* voor het transformeren van olierijke microalgen nog niet goed ontwikkeld is, wordt er in **hoofdstuk 7** gebruik gemaakt van de model soort *C. reinhardtii*. De zetmeelarme mutant BAFJ5 wordt gebruikt om aan te tonen dat het concept toepasbaar is op microalgen. In dit hoofdstuk werd het enzym dat stearinezuur omzet in oliezuur, stearoyl-ACP desaturase (SAD), onderdrukt door kunstmatige microRNAs. Twee verschillende constructen, specifiek voor twee verschillende posities op het mRNA van SAD, zijn getest. Voor elk construct werden drie transformanten gecultiveerd en de totale hoeveelheid vetzuren en TAG werd gemeten bij twee temperaturen gedurende het experiment. De mRNA niveaus van SAD werden verlaagd in de stammen die het silencing construct tot expressie brachten. In een van de stammen resulteerde de vermindering van het SAD mRNA in een verdubbeling van het stearinezuurgehalte in de TAG moleculen, waaruit blijkt dat het verhogen van de fractie van stearinezuur in TAG mogelijk is. Verder veronderstellen we dat naast de directe om-

zetting van oliezuur naar stearinezuur in de chloroplast, *C. reinhardtii* ook stearinezuur kan omleiden van de chloroplast naar het cytosol en in het endoplasmatisch reticulum alsnog stearinezuur kan omzetten in oliezuur met behulp van het enzym stearoyl-CoA desaturase.

In **hoofdstuk 8** wordt een vooruitblik gedaan op toekomstige strategieën om stamverbetering in microalgen te verbeteren gebaseerd op het werk beschreven in dit proefschrift. Daarnaast wordt er een routekaart beschreven om genetisch gemodificeerde microalgen en daarvan afgeleide producten op de markt te krijgen. In de nabije toekomst zal de stamverbetering van microalgen voornamelijk gebaseerd zijn op traditionele methodes zoals mutagenese, laboratorium evolutie en veredeling, waarvan ook in dit proefschrift voorbeelden gegeven worden. Een methode wordt voorgesteld voor het selecteren van mutanten met een verminderde weerbaarheid van de celwand en die meer toegankelijk zijn voor genetische modificatie. Zodra genetisch gemodificeerde (GG) microalgen met verbeterde eigenschappen zijn gemaakt, moeten ze door de regelgevende instellingen worden goedgekeurd om de productie ervan mogelijk te maken. Om GG microalgen in Europa op de markt te krijgen zullen die moeten voldoen aan de volgende kenmerken: een soort met de GRAS-status (generally regarded as safe, algemeen als veilig beschouwd), geteeld in gesloten fotobioreactoren in een kas. Het transgen dat is geïntroduceerd verbetert de productiviteit van een niet schadelijk product, bevat geen antibiotica resistentie gen en heeft een verminderde overlevingskans ten opzichte van wildtype soorten buiten de reactor.

De resultaten die worden beschreven in dit proefschrift zorgen voor een aanzienlijke verbetering in het begrijpen van TAG ophoping en koolstof allocatie in olierijke microalgen. Daarnaast zijn er verbeterde stammen gemaakt met een verhoogde TAG productiviteit of TAG vetzuur samenstelling. Bovendien wordt er een visie gepresenteerd waarin de belangrijkste knelpunten worden onderstreept in toekomstige industriële toepassingen van microalgen. Ik hoop dat met het werk dat wordt beschreven in dit proefschrift duurzame productie van (plantaardige) olie met microalgen een stap dichter bij verwezenlijking is.

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- Lenny -

About the Author

Lenny de Jaeger was born in Breda, The Netherlands, on the 28th of September 1985. He attended the Onze Lieve Vrouwelyceum in Breda and graduated in 2004. After high school he started his BSc in Biology at Wageningen University and specialized in molecular and cellular biology. After obtaining his BSc, he continued with an MSc in Biotechnology at Wageningen University and specialized in molecular biology and microbiology. During his MSc thesis he worked on the cultivation and genome analysis of sponge associated microorganisms in the marine sponge *Crambe crambe* at the chair group of microbiology at Wageningen University. For is internship he visited the Mayfield laboratory at the University of California San Diego, USA, where he conducted research on heterologous therapeutic protein expression in microalgae. In 2011 he completed his MSc studies and started his PhD research at the department of Bioprocess Engineering and Food and Biobased research both part of Wageningen UR. During his PhD research he worked on strain improvement of oleaginous microalgae for the production of a sustainable edible oil platform. Lenny was a Benelux finalist for the international science communication competition FameLab and chaired the committee that organized the first Young Algaeneers Symposium, which attracted more than 120 young microalgae scientists from over 22 different countries. Lenny is currently employed as Development Scientist in Innovation Compounding at Chr. Hansen A/S in Hørsholm, Denmark.

List of publications

Özturk, B., **de Jaeger, L.**, Smidt, H., and Sipkema, D. (2013). Culture-dependent and independent approaches for identifying novel halogenases encoded by Crambe crambe (marine sponge) microbiota. Scientific reports 3, 2780.

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Breuer, G.[‡], **de Jaeger, L.[‡]**, Artus, V.P., Martens, D.E., Springer, J., Draaisma, R.B., Eggink, G., Wijffels, R.H., and Lamers, P.P. (2014). Superior triacylglycerol (TAG) accumulation in starchless mutants of Scenedesmus obliquus: (II) evaluation of TAG yield and productivity in controlled photobioreactors. Biotechnology for biofuels 7, 70.
[‡]Authors contributed equally

de Jaeger, L., Carreres, B.M., Springer, J., Martens, D.E., Schaap, P.J., Eggink, G., and Wijffels, R.H. Neochloris oleoabundans is worth its salt: transcriptomic analysis under salt and nitrogen stress. *Submitted for publication.*

de Jaeger, L., Herpers, M., Springer, J., Wolbert, E.J.H., Martens, D.E., Eggink, G., and Wijffels, R.H. Gene silencing of Stearoyl-ACP desaturase enhances the stearic acid content in Chlamydomonas reinhardtii. *Submitted for publication.*

de Jaeger L., Lip, K.Y.F., Olijslager, J., Springer, J., Wolbert, E.J.H., Martens, D.E, Sturme, M.H.J, Eggink, G., and Wijffels, R.H. Transformation approach for novel microalgae. *To be submitted.*

Overview of completed training activities

Discipline specific activities

ESF Summer School (Bielefeld, Germany, 2011)
Microorganisms for Bio-fuel Production from Sunlight (Bielefeld, Germany, 2011)[1]
RNAseq Workshop (EMBL-EBI, Hinxton, UK, 2012)
International symposium on Algae for Energy (Qingdao, China, 2012)
JSTP China-Netherlands workshop (Beijing, China, 2012)
1st Young Algaeneers Symposium (Wageningen, 2012)[2]
2nd Young Algaeneers Symposium (Montpellier, France, 2014)[2]
Algal Biomass Organization (San Diego USA, 2014)[2]
Prospects and challenges for the development of algal biotechnology (Bielefeld, Germany, 2014)[2]

General courses

PhD Competence Assessment (Wageningen, 2009)
How to write a world class paper (Wageningen, 2011)
VLAG PhD week (Baarlo, 2011)
PhD scientific writing (Wageningen, 2012)
PhD trip network course (Wageningen, 2012)
Career Perspectives (Wageningen 2014)
Biobusiness Summerschool (Amsterdam, 2014)
Guide to scientific artwork (Wageningen, 2014)
BioBusiness Masterclass (HollandBio, 2014-2015)

Optionals

Bioprocess Engineering Brainstorm days (Wageningen, 2011-2015)[2]
Emerald oils Team Meetings (Wageningen and Vlaardingen, 2011-2015)[2]
1st International Young Algaeneers Symposium (2011-2012)[3]
Bioprocess Engineering PhD excursion Germany (2011)
Bioprocess Engineering PhD excursion Spain (2012)[1,2]

[1]Poster [2]Oral presentation [3]Organization

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