Engineering Increased Oil Productivity from Microalgae

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Zusammenfassung

Das Versiegen von Erdölreserven erfordert die Suche nach Alternativen. Diese bietet Biodiesel, ein aus Pflanzenölen (Triglyceriden, TAGs) gewonnener Biokraftstoff, der durch Umesterung mit einem einfachen Alkohol zu Biodiesel konvertiert werden kann. Dieser ist chemisch ähnlich zu herkömmlichen Diesel und bietet zusätzliche Vorteile gegenüber dem fossilen Brennstoff, da Biodiesel aus nachwachsenden Rohstoffen, wie aus ölhaltigen Pflanzen oder Algen gewonnen werden kann, und seine Produktion potentiell Kohlenstoff-neutral ist. Dabei bietet Biodiesel aus Mikroalgen weitere wesentliche Vorteile gegenüber dem aus Pflanzen, wie das wesentlich schnellere Wachstum oder den höheren Ölgehalt. Die Mikroalge *Chlorella vulgaris* hat sich dabei als vielversprechend für die Biodieselproduktion herausgestellt. Um die Öl-Produktivität und somit die Biodiesel-Ausbeute von Algen weiter zu steigern, wird auch in diesem Bereich die Gentechnik als wirksames Instrument herangezogen. Das Ziel dieser Studie war es, das potentiell limitierende Enzym für die TAG-Biosynthese zu identifizieren und charakterisieren, und ein Protokoll zur gentechnischen Veränderung von *C. vulgaris* zu etablieren.

Zunächst wurde die TAG-Biosynthese von *C. vulgaris* auf potenzielle Enzyme untersucht, welche die TAG-Produktion limitieren könnten. Hier wurde die Diacylglycerol Acyltransferase Typ-1 (DGAT1) identifiziert. Das *dgat1* Gen aus *C. vulgaris* (*Cvudgat1*; cDNA 1383 bp) wurde erfolgreich sequenziert. Bei der Charakterisierung des CvuDGAT1 Proteins (460 AS) wurde herausgefunden, dass dieses viele markante Sequenz-Motive und einige typische Merkmale mit anderen DGAT1 Proteinen aus Mikroalgen und Pflanzen teilt. Zum ersten Mal wurde ein Model der Tertiärstruktur von DGAT1 vorgestellt und dessen Einbettung in die ER-Membran bildlich dargestellt. Zusätzlich wurde das *Cvudgat1* Gen funktionell charakterisiert, indem es heterolog in einer Hefemutante exprimiert wurde, die kein Öl produzieren kann. Durch die Einbringung und Expression des rekombinanten *Cvudgat1* Gens wurde in dieser Hefe die Eigenschaft wiederhergestellt, Öl zu produzieren.

Für eine einfache, schnelle und effiziente Überexpression von gewünschten Genen wurde in einem zweiten Schritt ein vollständiges Protokoll zur gentechnische Veränderung von *C. vulgaris* erstellt. Hierfür wurden eine hocheffiziente Expressionskassette konstruiert und mehrere Transformations-methoden getestet. In dem Vektor pMDC162 wurden insgesamt vier Promotoren mit Hilfe des Reportergens ß-Glucuronidase in *Nicotiana benthamiana* und *Chlamydomonas reinhardtii* evaluiert. Dazu wurde der *Mannopine* S*ynthase* Promotor (MAS), der *Polyubiquitin-1* Promotor (Ubi1), das kombinierte Promotorsystem aus dem Hitzeschock Protein 70A und dem RuBisCO Promotor (Hsp70A/RBCS2) und der RBCS2 Promotor verwendet. In beiden Spezies zeigte der MAS und der Hsp70A/RBCS2 Promotor die stärkste Expression. Drei Transformationsmethoden für *C. vulgaris* wurden getestet, einschließlich Elektroporation, *Agrobacterium*-vermittelter Gentransfer und die Polyethylenglykol (PEG-) Methode.

Abschließend wurde das *Cvudgat1* Gen in die finale Expressionskassette mit einem hoch effizienten 5' - und 3' - untranslatierten Bereich im pMDC162 Vektor eingebracht, um das Protokoll zur gentechnischen Veränderung von *C. vulgaris* zu testen und die Auswirkungen der Expression des rekombinanten *Cvudgat1* Gens auf den *C. vulgaris* Öl-Stoffwechsel zu analysieren.

Schlagwörter: *Chlorella vulgaris*, Diacylglycerol Acyltransferase Typ-1, gentechnische Veränderung von Mikroalgen

Abstract

Crude oil reserves are dwindling dramatically and fuels derived from plant materials are an attractive source of energy. Those biofuels can be derived from plant oils (triacylglycerols, TAGs) by converting to biodiesel through transesterification with a simple alcohol. Biodiesel is chemically similar to conventional diesel, but have additional advantages over fossil fuels. For example, the primary feedstocks of biodiesel are oils from renewable sources, such as that found in oil seeds and oleaginous algae and its production is potentially carbon neutral. Hereby, microalgae provides several substantially advantages over plant derived biodiesel, including faster growth and higher oil yield. The microalgae *Chlorella vulgaris* exhibits a desirable biodiesel profile for biodiesel production with mainly saturated and monounsaturated fatty acids. With these properties *C. vulgaris* represents itself as a promising candidate for being a feedstock for the next generation biofuels. To further improve microalgal biosynthesis of biodiesel, genetic engineering is an effective tool, which gains increased importance in research. Therefore, our overall goal was to identify and characterize the potential bottleneck of TAG-production and to establish a complete genetic engineering protocol tailor-made for *C. vulgaris*.

During this thesis, we investigated the TAG-biosynthesis pathway of *C. vulgaris* for potential bottlenecks and identified the diacylglycerol acyltransferase type-1 (DGAT1) as rate-limiting enzyme for TAG-production. We successfully sequenced the *dgat1* gene from *C. vulgaris* (*Cvudgat1*; cDNA 1,383 bp) and found that the CvuDGAT1 protein (460 aa) shares many distinctive motifs and several typical characteristics with other DGAT1 sequences in microalgae and plants. In addition, we performed the first tertiary structure prediction of DGAT1 and its embedding into the ER membrane. We also functionally characterized *Cvudgat1* by expressing it heterologously in a quadruple disrupted non-oil-producing yeast strain. The expression of *Cvudgat1* restored TAG-production in the yeast mutant.

For an easy, rapid and efficient overexpression of desired genes, we established in a second step a complete genetic engineering protocol tailored for *C. vulgaris*. Therefore, we constructed a high efficient expression cassette and tested several transformation methods. We chose the pMDC162 vector and tested in total four promoters for evaluation in *Nicotiana benthamiana* and *Chlamydomonas reinhardtii* via ß-glucuronidase (GUS). The promoters include the mannopine synthase promoter (MAS), the polyubiquitin-1 promoter (Ubi1), the chimeric heat shock 70A – RuBisCO promoter system (Hsp70A/RBCS2) and the RBCS2 promoter alone. In both species, the MAS and the Hsp70A/RBCS2 promoter showed the strongest expression. To establish an efficient protocol to stably transform *C. vulgaris*, several transformation methods were tested, including electroporation, *Agrobacterium*-mediated gene transfer, and the PEG method.

In a final step, the *Cvudgat1* gene was inserted into the compiled final expression cassette, containing a highly-efficient 5´- and 3´- untranslated region (UTR), within the pMDC162 vector, ready to test the genetic engineering protocol and analyze the impact of the recombinant gene on the *C. vulgaris* oil-metabolism.

Keywords: *Chlorella vulgaris*, diacylglycerol acyltransferase type-1, genetic engineering of microalgae

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List of abbreviations

A	:	adenine
A. tumefaciens	:	*Agrobacterium tumefaciens*
aa	:	amino acids
ACAT	:	acetyl-CoA acetyltransferase
ACCase	:	acyl-CoA-carboxylase
ACP	:	acyl carrier protein
AICc	:	Akaike Information Criterion, corrected
amp	:	ampicillin
ASAT1	:	acyl-CoA sterol acyltransferase type 1
ASAT2	:	acyl-CoA sterol acyltransferase type 2
ASP	:	Aquatic Species Program
ASTM	:	American Society for Testing & Materials
ATP	:	adenosin triphosphat
Bodipy	:	4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene
BBM	:	Bold´s Basal Media
bp	:	base pair
C	:	cytosine
C. vulgaris	:	*Chlorella vulgaris*
C. reinhardtii	:	*Chlamydomonas reinhardtii*
C-terminus	:	carboxy-terminus
CaMV 35S	:	virus-derived Cauliflower Mosaic Virus 35S promoter
cDNA	:	complementary deoxyribonucleic acid
CO_2	:	carbon dioxide
CTAB	:	cetrimonium bromide
Cvudgat1	:	*dgat1* gene from *C. vulgaris*
CvuDGAT1	:	DGAT1 protein from *C. vulgaris*
DAG	:	diacylglycerol
DAS	:	dense alignment surface
DCW	:	dry cell weight
DGAT1	:	diacylglycerol acyltransferase **type-1**
DGAT2	:	diacylglycerol acyltransferase **type-2**
DGAT3	:	diacylglycerol acyltransferase **type-3**
DHAP	:	dihydroxyacetone phosphate
DMSO	:	dimethyl sulfoxide
dATP	:	deoxyadenosine triphosphate
dCTP	:	deoxycytidine triphosphate
dGTP	:	deoxyguanosine triphosphate
DNA	:	deoxyribonucleic acid
dNTPs	:	deoxyribonucleotide (including dATP, dTTP, dGTP and dCTP)
DO-mix	:	drop-out mix

DOB-media	:	dropout base media
DSMZ	:	"*Deutsche Sammlung von Mikroorganismen und Zellkulturen*" German Collection of Microorganisms and Cell Cultures
dTTP	:	deoxythymidine triphosphate
E. coli	:	*Escherichia coli*
EDTA	:	ethylenediaminetetraacetic acid
ES	:	"erddekokt" and salt
ER	:	endoplasmic reticulum
EtBr	:	ethidium bromide
EV	:	electric volume
FA	:	fatty acid
FAD2	:	atty acid desaturase type-2
FAME	:	fatty acid methyl ester
FC	:	flow cytometry
Fe^{3+}	:	ferric oxide
FCW	:	fresh cell wight
FL1	:	first fluorescence detector
G	:	guanine
G418	:	gentamycin
G3P	:	glyceraldehyde 3-phosphate
G3PDH	:	glyceraldehyde 3-phosphate dehydrogenase
GAL1	:	galactose-inducible hybrid promoter
GC-MS	:	gas chromatography mass spectrometry
gDNA	:	genomic deoxyribonucleic acid
gfp	:	green-fluorescent-protein gene
goi	:	gene of interest
gus	:	ß-glucuronidase gene
GPAT	:	*sn*-glycerol-3-phosphate acyltransferase
GWsys	:	Gateway system
H_2O	:	water
hptII	:	hygromycin phosphotransferase II gene
Hsp70A	:	heat shock protein 70A promoter
Hsp70A/RBCS2	:	Hsp70A/RBCS2 chimeric promoter system
hygB	:	hygromycin B
IPTG	:	isopropyl β-D-1-thiogalactopyranoside
JTT	:	Jones-Taylor-Thornton model
kana	:	kanamycin
KAS	:	3-ketoacyl-ACP synthase
kb	:	kilo base
KD	:	Kyte & Doolittle
kDa	:	kilodalton
L-PtdOH	:	lyso-phosphatidic acid

LANL	:	Los Alamos National Laboratory
LB	:	left border
LB-media	:	lysogeny broth media
LEC1	:	LEAFY COTYLEDON1 transcription factor
LEC2	:	LEAFY COTYLEDON2 transcription factor
LiAc	:	lithium acetate
LLD	:	lower level discriminator
LPA	:	lysophosphatidic acid
LPAAT	:	lyso-phosphatidic acid acyltransferase
luc	:	luciferase gene
LUH	:	Leibniz University Hanover (Germany)
lyso-PC	:	lyso-phosphatidylcholines
MAS	:	mannopine synthase promoter
MBOAT	:	membrane bound O-acyltreanserase
mRNA	:	messenger ribonucleic acid
MS-media	:	Murashige & Skoog media
N. benthamiana	:	*Nicotiana benthamiana*
N-terminus	:	amino-terminus
NCBI	:	National Center for Biotechnology Information
NEB	:	New England BioLabs
NEU	:	Northeastern University Boston (USA)
nos	:	nopaline synthase
nptII	:	neomycin phosphotransferase II gene
nt	:	nucleotide
OD	:	optical density
OE-PCR	:	overlap-extension-PCR
PBS	:	phosphate buffered saline
PC	:	phosphatidylcholines
PCR	:	polymerase chain reaction
PDAT	:	phospholipid:diacylglycerol acyltransferase
PEG	:	polyethylene glycol
Phe	:	phenylalanine
PMT	:	photomultiplier
PP	:	phosphatidic acid phosphatase
PsaD	:	photosystem I subunit promoter
PtdOH	:	phosphatidic acid
RB	:	right border
RBCS2	:	promoter of the small subunit 2 of ribulose-1,5-bisphosphate carboxylase / oxygenase
RE sites	:	recognition sites for restriction enzymes
RNA	:	ribonucleic acid
RT	:	room temperature

RT-PCR	:	real time (quantitative) PCR
RuBisCO	:	ribulose-1,5-bisphosphate carboxylase / oxygenase
S. cerevisiae	:	*Saccharomyces cerevisiae*
SAP	:	shrimp alkaline phosphatase
SC-media	:	*Saccharomyces cerevisiae* media
SH-media	:	Schenk & Hildebrandt media
SiC	:	silicon carbide
SOB-media	:	super optimal broth media
SOC-media	:	super optimal broth with catabolite repression media
spec	:	spectomycin
STM	:	SHOOT MERISTEMLESS transcription factor
T	:	thymine
TAE	:	tris - acetat - EDTA
TAG	:	triacylglycerol
TCC	:	total cell count
TE	:	tris EDTA
TF	:	transcription factor
T_i	:	tumor inducing
TMD	:	transmembrane domains
tris	:	tris (hydroxymethyl) aminomethane
TY-media	:	trypton yeast media
u	:	unit
rpm	:	rounds per minute
Ubi1	:	polyubiquitin-1 promoter
UTEX	:	The University of Texas at Austin (USA)
UTR	:	untranslated region
v/v	:	volume per volume
w/o	:	without
w/v	:	weight per volume
WRI1	:	WRINKLED1 transcription factor
x-gal	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
x-gluc	:	5-bromo-4-chloro-1*H*-indol-3-yl β-D-glucopyranosiduronic acid
YEP-media	:	yeast-peptone media
yfp	:	yellow-fluorescent-protein gene
YPD-media	:	yeast-peptone-dextrose media

1. Introduction

Since the world's petroleum is limited and will suffice for a few more decades, researchers around the world are looking for sustainable alternatives (detailed in Chapter 2, Section 2.1.). To address the dwindling oil reserves, an attractive alternative is plant-derived biofuels. This field of research started in the 1930s and developed to a significant sector of industry (Huang *et al.* 2010). Currently, plant-produced transportation fuels, such as bioethanol or biodiesel, are derived mainly from food crops, such as corn, oilseed rape, and soybeans (Scott *et al.* 2010; Demirbas 2007). However, the yield will never supply the global consumer´s demand (Chisti 2007), which also involves the *food vs. fuel* dispute (Yu *et al.* 2011; Wijffels and Barbosa 2010; Scott *et al.* 2010; Chisti 2007; detailed in Chapter 2, Section 2.2.).
In the last decade, microalgae were considered a promising alternative feedstock for the next generation biofuels. Microalgae have higher oil-content and faster reproduction rate than plants (Chen and Smith 2012; Demirbas and Fatih Demirbas 2011; Scott *et al.* 2010; Mata *et al.* 2010; Wijffels and Barbosa 2010), and are flexible in cultivation regarding to its undemanding growing conditions and easy handling (Rasoul-Amini *et al.* 2011; Demirbas and Fatih Demirbas 2011; detailed in Chapter 2, Section 2.3.).

In this thesis, we are concentrating on improving the lipid productivity of the microalgae *Chlorella vulgaris* (Beyernick 1890) by genetic engineering.
C. vulgaris is a small, unicellular green alga, which is well explored (Scragg *et al.* 2003; Illman *et al.* 2000; Hawkins and Nakamura 1999). Its doubling time was determined to 9 - 14 hours under optimal growing conditions (Hawkins and Nakamura 1999) and its biomass productivity can reach up to 200 mg fresh cell weight (FCW) per liter per day (Mata *et al.* 2010). The average oil-content in its natural environment was found to be about 18 % (Illman *et al.* 2000), but can be further increased by optimizing its growing conditions and by applying stress (detailed in Chapter 2, Section 2.4.1.). Furthermore, the fatty acid methyl ester (FAME) composition in *C. vulgaris* with mainly saturated fatty acids (FAs) and a high amount of monounsaturated FAs is a desirable profile for biodiesel (Praveenkumar *et al.* 2012; Hobuss *et al.* 2011; Guarnieri *et al.* 2011; Kurt 2011; Zheng *et al.* 2008; Rashid *et al.* 2008; detailed in Chapter 2, Section 2.4.). Due to these properties, *C. vulgaris* is highly valued as candidate for commercial biodiesel production (Mata *et al.* 2010; Lv *et al.* 2010).

Biodiesel is derived from triacylglycerols (TAGs), also known as fats or oils. These are one of the energy richest carbon forms found in nature and are used in various organisms as energy storage compound. TAGs consist of three FA chains commonly with a length of 16 to 18 carbons esterified via the hydroxyl groups to one molecule glycerol (Durrett *et al.* 2008). Oil-biosynthesis occurs in two major steps (detailed in Chapter 2, Section 2.5): (1) the FAs are synthesized in the chloroplast by the FA synthesis complex. The FAs are then transported to the endoplasmic reticulum (ER), where (2) the Kennedy- or TAG-pathway takes place. Here, the FAs are assembled to the glycerol molecule to form TAG (Liu and Benning 2013; Yu *et al.* 2011). The key enzymes for the oil-biosynthesis were

determined to be acyl-CoA-carboxylase (ACCase) and 3-ketoacyl-ACP synthase (KAS) from the FA synthesis pathway (Dehesh *et al.* 2001; Dunahay *et al.* 1996) and *sn*-glycerol-3-phosphate acyltransferase (GPAT), lyso-phosphatidic acid acyltransferase (LPAAT), and diacylglycerol acyltransferase (DGAT) from the *Kennedy pathway* (Radakovits *et al.* 2010; Yu *et al.* 2011; detailed in Chapter 2, Section 2.5.).

To overcome those potential bottlenecks of biodiesel production, research groups around the world are increasing the TAG-biosynthesis level in microalgae by genetic engineering. The main crucial step, therefore, is the stable integration of recombinant genes into the algal genome. Another hurdle, thereby, is the moderate expression level determined for the synthesis of foreign genes in algae (León and Fernández 2007; León-Bañares 2004; Chen *et al.* 2001; detailed in Chapter 2, Section 2.8.). Motivated by this challenge, we established a highly-efficient genetic engineering protocol for the microalga *C. vulgaris*.

The main aim of this thesis was to genetically engineer the microalga *C. vulgaris* by overexpression of the potential bottleneck diacylglycerol acyltransferase type-1 (DGAT1, further discussed in the following) to investigate its impact on TAG-production. Therefore, we pursued two aims:

Aim 1: The identification and characterization of DGAT1 from *C. vulgaris*, including structural analysis of the DGAT1 protein through bioinformatic tools and its functional characterization in non-oil-producing yeast strains (Chapter 4)

Aim 2: The establishment of a complete genetic engineering protocol, containing the construction of a highly-efficient expression cassette tailor-made for *C. vulgaris* and the optimization of a suitable and efficient transformation method for this algal (Chapter 5)

We initiated this study with the investigation of potential bottlenecks of TAG-production. The DGAT type-1 enzyme represents a potential limiting enzyme for oil-production in *C. vulgaris*. In a next step, we explored its sequence encoded by *C. vulgaris*, but also in its entire functionality and impact range on TAG productivity (Chapter 4).

DGAT is the enzyme, which catalyzes the last step in TAG-biosynthesis (Kennedy 1961). In total, three DGAT enzymes have been identified in microalgae and plants, DGAT1 to DGAT3, and all of them seem to play a role in TAG-production, whereas, DGAT1 and DGAT2 act in a major role. The two proteins are located within the ER membrane (Guihéneuf *et al.* 2011). Even when both enzymes catalyze the same reaction, they do not show sequence similarities (Chen and Smith 2012; La Russa *et al.* 2012; Lung and Weselake 2006). DGAT1 and DGAT2 were found in a wide range of eukaryotic organisms, including humans, vertebrates, higher plants, but also in fungi, lower animals, and microalgae. Thereby, the preference to use DGAT1 or DGAT2 is species-dependent (Shockey *et al.* 2006). In several studies, it has been demonstrated that the DGAT1 enzyme plays the major role for TAG-production in *C. vulgaris* (Guarnieri *et al.* 2011; Miller *et al.* 2010; detailed in Chapter 2, Section 2.7.).

In this study, we successfully identified the *dgat1* gene from *C. vulgaris* (*Cvudgat1*). Through bioinformatics, we found that CvuDGAT1 shares typical characteristics and for its enzymatic function

important sequence motifs with other DGAT1 sequences in microalgae and plants. In addition, we constructed the putative tertiary structure of DGAT1 and predicted its embedding into the ER membrane (detailed in Chapter 4, Section 4.3.3.). Then, we functionally characterized *Cvudgat1* by expressing it heterologously in a quadruple disrupted non-oil-producing yeast mutant (detailed in Chapter 4, Section 4.3.4.). With this, we verified that the isolated gene from *C. vulgaris* has DGAT enzyme activity in yeast and is involved in TAG-biosynthesis.

In the second part of this thesis, we constructed a highly-efficient expression system tailor-made for *C. vulgaris* and tested several transformation methods for successful integration of recombinant DNA into the algal genome. The final aim was to establish an efficient genetic engineering protocol for *C. vulgaris*, allowing us to overexpress any desired *gene of interest* (*goi*) easily and rapidly (Chapter 5).

To construct a highly-efficient vector for recombinant gene expression in microalgae, several factors have to be considered. The choice of the right vector, including a suitable selection system and the choice of a strong promoter, is important (Vila *et al.* 2012; León and Fernández 2007; Schroda *et al.* 2000). In the green alga *Chlamydomonas reinhardtii,* it has been demonstrated that most foreign promoters result in poor expression levels (Neupert *et al.* 2012). Currently, a common and well-studied promoter fusion for *C. reinhardtii* is the heat shock protein 70A promoter (Hsp70A) fused downstream to the promoter of the small subunit-2 of ribulose-1,5-bisphosphate carboxylase / oxygenase (RBCS2; Schroda *et al.* 2000; Strenkert *et al.* 2013; Eichler-Stahlberg *et al.* 2009; Li and Tsai 2009; Chen *et al.* 2008; detailed in Chapter 2, Section 2.8.1.).

In this thesis, we used four different promoters, including the common Hsp70A/RBCS2 chimeric promoter system, the RBCS2 promoter alone, the polyubiquitin-1 (Ubi1) promoter (resulting in good expression levels in *Chlorella ellipsoidea;* Chen *et al.* 2001), and the mannopine synthase (MAS) promoter, which has not been applied in microalgae. The promoters were evaluated via ß-glucuronidase (GUS) in *Nicotiana benthamiana* and *C. reinhardtii*.

The most challenging step for engineering microalgae is the transformation and stable integration of the transgene into the algal genome. For every microalgae species, the transformation has to be optimized, as the transformation efficiency is strongly species-dependent (Radakovits *et al.* 2010; Hallmann 2007). Several methods are commonly used to genetically modify microalgae, including the glass-bead method (Kindle 1990), which is similar to the PEG method (Jarvis and Brown 1991), the silicon carbide (SiC) method (Dunahay 1993), electroporation (Maruyama *et al.* 1994), particle bombardment (Mayfield and Kindle 1990), and the *Agrobacterium*-mediated gene transfer (Kumar *et al.* 2004; detailed in Chapter 2, Section 2.8.2.). For the *Chlorella* species, the most frequently applied transformation method is the electroporation (Maruyama *et al.* 1994; Huang *et al.* 2006; Chen *et al.* 2001; Chow and Tung 1999) and the PEG-method (Kim *et al.* 2002; Huang *et al.* 2006; Hawkins and Nakamura 1999).

In this thesis, to find a suitable highly efficient transformation method for creating stable transformants of *C. vulgaris*, we tested several methods, including electroporation, the PEG-method, and the *Agrobacterium*-mediated gene transfer (Chapter 5, Section 2.8.2.).

In summary, we successfully identified and characterized the *dgat1* gene from *C. vulgaris,* demonstrated that CvuDGAT1 has DGAT1 activity, and is involved in TAG-biosynthesis. We further found that CvuDGAT1, indeed, could represent the limiting enzyme in oil-production in *C. vulgaris*. To reveal CvuDGAT1 impact in oil synthesis in this alga, we constructed a genetic engineering protocol for *C. vulgaris*. With this engineering system, the *Cvudgat1* gene was intended to be overexpressed in *C. vulgaris* not only to verify the protocol, but also to measure the impact of *Cvudgat1* overexpression on its TAG-biosynthesis.

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2. Critical Literature Review

Microalgae represent themselves as strong candidates for biodiesel production and showed to be a promising alternative to petroleum fuels. They exhibit several advantages over conventional fuels and plant derived biodiesel, so microalgae derived biofuels likely will achieve a high impact on the global marked in the near future. In the present thesis, we focus on the microalgae *Chlorella vulgaris*, where the first part deals with the investigation of a potential bottleneck of the oil-biosynthesis pathway to illuminate its impact on oil-productivity. In the second part, we aimed to develop a comprehensive engineering protocol to enable the investigation of biosynthetic enzymes involved in oil-biosynthesis of *C. vulgaris*.

2.1. Energy Crisis and Alternative Sources

Peak oil and fading oil resources will occur in our lifetime. Currently, the requirements for global energy is anticipated to double from 12 TW/348 quadrillion BTU (1990) to 23 TW/687 quadrillion BTU (2030). Reasons for this increased energy demand are a growing world population, as well as increasing living standards (La Russa *et al.* 2012). The consumption of oil in turn increases atmospheric greenhouse gases. To satisfy the energy demand of the world in the future and to decrease further accumulation of greenhouse gases, several projects aim to contribute to the production of biofuels derived from plants and microalgae as an attractive alternative to petroleum fuel (Radakovits *et al.* 2010; Scott *et al.* 2010).

Research on biofuels derived form vegetables oils started in the 1930s and 1940s. In the meantime, the rapid development of the global industry and the increase in energy consumption made the research in the field of alternative energy sources inevitable (Huang *et al.* 2010). The term “biodiesel” describes fuels that are chemically converted from oil derived from plants, animal fats, and other renewable sources, such as microalgae. Biodiesel consists of monoalkyl esters formed by the catalyzed reaction of triacylglycerols (TAGs; oil or fat) with a simple alcohol such as methanol (Figure 2.1). These esters are known as biodiesel or fatty acid methyl esters (FAME; Demirbas and Demirbas 2011; Chisti 2007; Knothe 2005; Gerpen 2005).

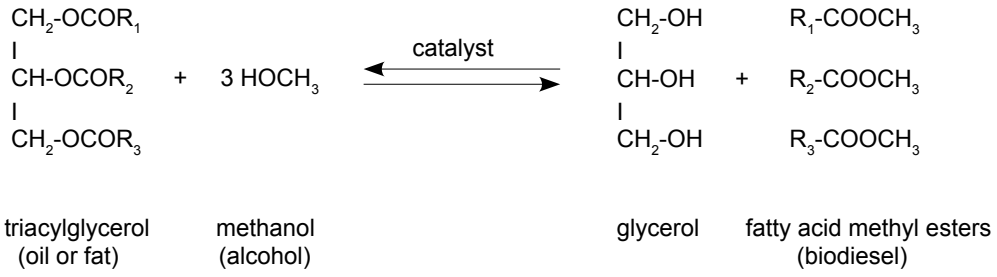

Figure 2.1 | Transesterification reaction of triacylglycerol or oil to biodiesel with a simple alcohol. R1–3 are hydrocarbon groups.

2.2. Biodiesel vs. Petroleum Fuels

Biodiesel is highly similar to petroleum fuels also regarding to the number of carbons in the FAMEs. While conventional diesel contains about 15 carbons, the chains of biodiesel consists of 14 to 18 carbons. This structural similarity of diesel oil and biodiesel makes it feasible to substitute petroleum fuel (Huang *et al.* 2010). However, biodiesel offers several advantages over the conventional petroleum fuels. For example, the primary feedstocks of biodiesel are photosynthetic organisms, which use sunlight and fix carbon dioxide (CO_2) from the atmosphere to produce oil. In this way, the production of the biofuels is not only carbon neutral, but also renewable (Merchant *et al.* 2012; Durrett *et al.* 2008). Furthermore, biodiesel has a higher flashpoint than the conventional diesel, as it contains more oleic acid and is therefore easier to handle and to store in huge amounts (Yu *et al.* 2011; Rashid *et al.* 2008; Bozbas 2008; Knothe 2005). As biodiesel is derived from natural sources, it is biodegradable, less toxic and environmentally friendly while reducing most regulated exhaust emissions in comparison to petroleum fuels. Due to its greater lubricity it prolongs engine lives, reduces the need for maintenance and shows a better efficiency than conventional diesel (Yu *et al.* 2011; Bozbas 2008; Rshid *et al.* 2008; Demirbas 2007). A main disadvantage is, however, the generally high costs for production, especially for the harvest and extraction processes, which are not solved yet (Huang *et al.* 2010). But, as in the near future the prices for petroleum fuels will further rise, biofuel production requires additional investigations in many areas of this field including not only to lower the production costs, but also to increase the yield from living organisms (Demirbas 2007).

Currently, plant derived biodiesel is mainly produced from corn, canola (oilseed rape) and soybeans (Scott *et al.* 2010; Demirbas 2007). However, with a closer consideration it becomes evident, that food crops cannot meet the needs of biodiesel. For example, the coverage of the biodiesel demand for one half of the existing U.S transport fuel will require a cropping area sized half of the surface area of the U.S., when calculating with the average oil yield per hectare from various crops (Chisti 2007). By consideration of this example it becomes obvious, that using plants for biodiesel production will arise a *food vs. fuel* dispute, entailing the question `Which industry can claim the restricted arable land for production, the food or the fuel industry?`. With this question it is inevitable that the industries will increase the food prices (Yu *et al.* 2011; Wijffels and Barbosa 2010; Scott *et al.* 2010). Another argument for not growing food crops for biofuel production is their high water requirement and the long growing time until the crop can be harvested to obtain the fuel (Yu *et al.* 2011).

A promising alternative to crop plants for biodiesel production are microalgae, which have several advantages over biodiesel derived from food crops. Firstly, microalgae can, like higher plants, convert sunlight into chemical energy in form of oils and carbohydrates. The more effective a plant can convert the solar energy, the higher is the yield by time for those energy storage compounds. Microalgae are the photosynthetically most efficient plants on earth, which entails a faster growth and higher oil yield than archived by crop plants (Chen and Smith 2012; Demirbas and Demirbas 2011; Scott *et al.* 2010; Huang *et al.* 2010; Mata *et al.* 2010; Wijffels and Barbosa 2010). Indeed, microalgae are the fastest-growing plants in the world (Demirbas and Demirbas 2011). Some species are able to reach a

biomass productivity of up to 7.7 g/L/day (*Euglena gracilis*, Mata *et al.* 2010). The oil yield per acre obtained by microalgae can be several times higher than those achieved from terrestrial plants (Demirbas and Demirbas 2011; Mata *et al.* 2010; Huang *et al.* 2010; Wijffels and Barbosa 2010). A comparison of oil yields achieved from various plants and microalgae in liter per hectare are shown in Figure 2.2.

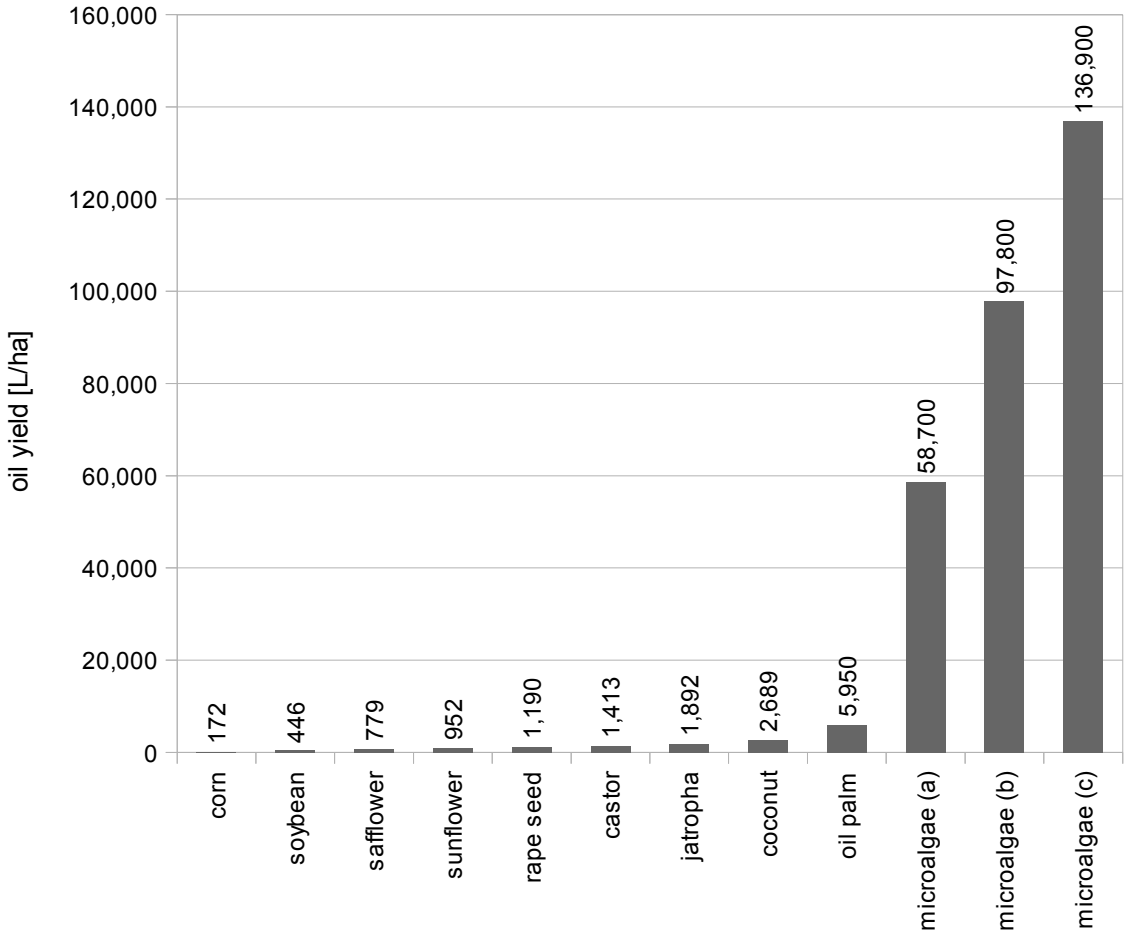

Figure 2.2 | Oil yield of various plants and microalgae. Microalgae labeled with an (**a**) considers species with a low oil-content, (**b**) species with a medium lipid productivity, and (**c**) high oil producing microalgae. This list was compiled from data reviewed by Demirbas and Demirbas 2011, Mata *et al.* 2010, and Chisti 2007.

Another significant benefit of microalgae is the given fact that they grow almost everywhere once there is sufficient light (Demirbas and Demirbas 2011). Therefore, microalgae can also be cultivated in deserts (Merchant *et al.* 2012; Wijffels and Barbosa 2010) or in cold climatic regions (Demirbas and Demirbas 2011). With this property, microalgae culturing is independent of arable land and makes the *fuel vs. fuel* debate for biodiesel production obsolete (Merchant *et al.* 2012; Wijffels and Barbosa 2010). Furthermore, considering the worldwide fresh water scarcity it is important that biofuels derived from microalgae need less water than when extracted from food plants. To produce 1 Liter of biodiesel, food crops will require about 10,000 Liter water, whereas microalgae need much less water. Approximately 0.75 Liter water is needed to produce one kilogram of biomass alone for biosynthesis (Wijffels and Barbosa 2010).

With these advantages over food crops, microalgae are representing themselves as strong candidates for the next generation biofuels. Therefore, the current research on sustainable biofuels become more and more focused on microalgae (La Russa *et al.* 2012; Rasoul-Amini *et al.* 2011; Yu *et al.* 2011; Radakovits *et al.* 2010; Scott *et al.* 2010; Wijffels and Barbosa 2010; Miller *et al.* 2010; Rodolfi *et al.* 2009).

2.3. Microalgae as "Biofuel Factory"

Microalgae started to gain attention as potential biofuel producer in the 1970s when the first oil crisis began. The U.S. started large research programs with the focus on the development of renewable energies from microalgae. Between 1978 and 1996, the *Aquatic Species Program* (ASP) was funded by the *U.S. Department of Energy´s Office of Fuels Development* to develop renewable fuels from high-oil producing microalgae. Among other results, the scientists involved published a list of algae strains, which could be promising candidates for biofuel production. This field is still an essential part of research these days (Wijffels and Barbosa 2010).

Microalgae can be described as sunlight driven cell-factories, which are able to convert CO_2 with solar energy to energy-rich products, such as oils, polyunsaturated fatty acids (FAs), carotinoids, sugars, pigments, antioxidants, foods, feed and high-value bio-active compounds (Mallick *et al.* 2012; Demirbas and Demirbas 2011; Mata *et al.* 2010; Chisti 2007). To convert microalgal CO_2 fixation efficiency into numbers: for the production of 1 ton of algal biomass up to 2 tons of CO_2 are needed (Mallick *et al.* 2012; Wijffels and Barbosa 2010; Chisti 2007). With this high efficiency and the amount of microalgae occurring on this planet, they are the organisms, which implement the most carbons with about 50 % of the global organic carbons (León and Fernández 2007; Field 1998). Furthermore, microalgae are the fastest-growing plants on earth (Mallick *et al.* 2012; Demirbas and Demirbas 2011). With respect to the growing conditions, microalgae are relatively unpretentiously and flexible, as they can almost grow everywhere when there is enough sunshine, including deserts (Merchant *et al.* 2012; Wijffels and Barbosa 2010), but also cold climatic regions (Demirbas and Demirbas 2011). For an effective growth the only essential elements needed are nitrogen, phosphorus, iron and for some species silicon (Chisti 2007). Additionally, some species can also be cultivated in seawater or wastewater (Merchant *et al.* 2012; Demirbas and Demirbas 2011; Wijffels and Barbosa 2010; Chinnasamy *et al.* 2010; Radakovits *et al.* 2010). Moreover, microalgae are able to live not only in phototrophical conditions, but also in mixo- and heterotrophical conditions and to use other environmental carbon sources than CO_2, such as glucose or glycerol (Belotti *et al.* 2013; Mallick *et al.* 2012; Liang *et al.* 2009; Zaslavskaia *et al.* 2001). Additionally, microalgae are also suitable for cultivation in bioreactors (Demirbas and Demirbas 2011; Zaslavskaia *et al.* 2001). All these given properties define the enormous potential of microalgae for widely varying applications.

Particularly for their application as biofuel producers, microalgae bring along several distinctive advantages:

- As microalgae can convert sunlight highly-efficient, many species can accumulate large quantities of oil (Demirbas and Demirbas 2011; Huang *et al.* 2010; Wijffels and Barbosa 2010; Rodolfi *et al.* 2009; Hu *et al.* 2008) with an average of 1 % and 70 % dry cell weight (DCW; Mata *et al.* 2010).
- The oil-productivity can easily be further increased by altering the culture conditions from phototrophic to mixo- or heterotrophic (Al-Iwayzy *et al.* 2014; Praveenkumar *et al.* 2012; Mallick *et al.* 2012; Mujtaba *et al.* 2012; Kurt 2011; Yeh and Chang 2011; Guarnieri *et al.* 2011; Miller *et al.* 2010; Converti *et al.* 2009; Liu *et al.* 2008; Illman *et al.* 2000) with up to 90 % oil DCW (Mata *et al.* 2010; detailed in Section 2.4.1).
- Algae biofuels contain high amounts of saturated FAs (mostly C16 and C18) and oleic acids (C18:1), which increases the oxidative stability for a longer and safer storage of its fuel (Mallick *et al.* 2012; Knothe 2005).
- The entire cell can be exploited. After extracting the oil, the rest of the microalgae can be converted to bio-ethanol or be used as animal feed (Mallick *et al.* 2012; Demirbas and Demirbas 2011; Wijffels and Barbosa 2010). The high-quality glycerol obtained as by-product of transesterification can be sold to industry or reused as additional carbon source in microalgae cultures (Demirbas 2007; Liang *et al.* 2009).
- As the vessel construction for microalgae cultures can be built upwards, an even high per-acre yield can be achieved (Demirbas and Demirbas 2011; Wijffels and Barbosa 2010).
- Caused by the high growth-rate, microalgae can be harvested more frequently (e. g. 1 - 3 doubling per day; Hu *et al.* 2008).

Nevertheless, microalgae show also some drawbacks when used for biodiesel production, as lipid productivity is species-dependent (Mata *et al.* 2010). This requires to select the right strain for each purpose. Microalgae can be separated into two groups: The first group consists of algae with a high lipid content but a low growth rate, while the second group includes microalgae with high growth rates but with a relatively low lipid content (Lv *et al.* 2010). For example, the alga *Botryococcus braunii* shows a lipid content of up to 75 % DCW but a volumetric biomass productivity of just 20 mg/L/d. In contrast, *Spirulina maxima* exhibited a growth rate up to 250 mg/L/d but has a lipid content of only 4 - 9 % DCW (Mata *et al.* 2010). As a second drawback, it should be considered that biodiesel production from microalgae is still a relative new technology (Demirbas and Demirbas 2011). Several steps in harvest and oil extraction have to be optimized (Chisti 2007). For example, the main limiting factor for an efficient application of microalgae as biofuel feedstock is the high energy costs for processing of the oil. Presently, the harvest of microalgae is performed by centrifugation, which requires high energy. An alternative could represent an algal flocculation step prior to centrifugation, which would significantly shorten the centrifugation step. But this technology needs a further improvement for being applied commonly in industries (Wijffels and Barbosa 2010). This is reflected

by comparing the market prices for microalgae to those of oil plants: In 2010, microalgae had a market volume of in total 1.25 billion € with an average market price of 250 €/kg, in contrast, palm oil with a world production of almost 40 million tons had a market value of about 0.50 €/kg (Wijffels and Barbosa 2010).
In order to develop microalgae derived biofuels competing in the global market two goals should be approached to lower market prices: (1), the processing steps have to be made less energy demanding; (2) an efficient market system for the trade of by-products, such as the high-quality glycerol, should be established (Demirbas 2007). An additional opportunity could be a genetically modified high oil-producing algae strain for a higher oil yield.

To conclude this section, microalgae represents themselves as promising candidates for biofuel production as an alternative the fading fossil fuels but also for terrestrial oleaginous plants (Yu *et al.* 2011). To obtain an effective and low cost feedstock in this technology it still needs significant improvements (Chisti 2007). These include especially the harvest and extraction process, but also the understanding of the algal metabolic pathways and – most important – the genetic engineering to optimize algal properties (Huang *et al.* 2010; Radakovits *et al.* 2010; Yu *et al.* 2011; Chisti 2007).

2.4. Why Chlorella vulgaris?

Current research on microalgae for biodiesel production is focused on a small number of fast-growing species. In the group of the green algae the main focus is on *Chlamydomonas reinhardtii*, *Dunaliella salina* and various *Chlorella* species (Scott *et al.* 2010). *Chlorella vulgaris* has already been investigated for its lipid content. Gas chromatography-mass spectrometry (GC-MS) investigations of the lipid content revealed that *C. vulgaris* contains mainly saturated FAs (C16:0 and C:18:0; 82 % of total FAME) and a high amount of monounsaturated oleic acid (C:18:1 Δ^9; Praveenkumar *et al.* 2012; Hobuss *et al.* 2011; Guarnieri *et al.* 2011; Kurt 2011; Zheng *et al.* 2008; Rashid *et al.* 2008), which lowers the freezing point of its biodiesel (Ramos *et al.* 2009) and increase its oxidative stability (Mallick *et al.* 2012; Rasoul-Amini *et al.* 2011). With this FAME composition, the biodiesel profile of *C. vulgaris* matches the standards laid down by the *American Society for Testing and Materials* (ASTM) EN 14 214 and by the Indian IS 15 607 (Al-Iwayzy *et al.* 2014; Mallick *et al.* 2012; Rasoul-Amini *et al.* 2011), which consider physical properties of a fuel, such as density, viscosity, acid value, iodine value, calorific value, cetane index, ash, and water contents (Al-Iwayzy *et al.* 2014). These promising properties of *C. vulgaris* makes it to a suitable candidate for research to increase its biodiesel productivity by genetic engineering.

C. vulgaris itself is classified as *chlorophyta* (phylum) and is a unicellular green alga with a size between 3 - 10 µm (Scragg *et al.* 2003; Illman *et al.* 2000). As first isolated and cultivated photosynthetic microalgae in 1890 (Beyernick 1890) *C. vulgaris* is well-explored. Its doubling time were determined to 9 - 14 hours under optimal growing conditions (Hawkins and Nakamura 1999) with a volumetric biomass productivity of up to 200 mg fresh cell weight (FCW) per liter per day (Mata *et al.*

2010). Under normal growing conditions *C. vulgaris* consists of ~ 29 % protein, ~ 51 % carbon hydrates and ~ 18 % lipids (Illman *et al.* 2000). The composition can highly be influenced by stress (Section 2.4.1.). The metabolic pathways were determined to share high similarities to those from higher plants, such as expression patterns, post-translational modifications and glycosylations (Chen *et al.* 2001). The DNA from *C. vulgaris* contain mainly guanine (G) and cytosine (C) with a GC-content of 62.3 % (Kessler 1976), which is similar to other microalgae such as *C. reinhardtii* with 65 % GC-content (Mayfield and Kindle 1990). Due to its thick and tough cell wall *C. vulgaris* is very resistant to external influences (Radakovits *et al.* 2010; Huang *et al.* 2006). For example, the acidic limit for growth was determined to a pH of 4 and its salt tolerance reaches up to an environmental salt concentration of 4 % (Kessler 1976). Furthermore, it has been found that *Chlorella* is one out of only three taxa (*Spirulina*, *Dunaliella* and *Chlorella*) suitable for sustained open pond cultivation systems for commercial production, as these were the only species out of hundreds tested in which the contamination did not take over in open pond systems (Huntley and Redalje 2007). Next to the feasibility to grow *C. vulgaris* in large ponds, this microalgae has been shown to be suitable for cultivation in different kinds of photobioreactors (Fan *et al.* 2008).

In summary, *C. vulgaris* can be regarded as promising candidate for commercial biodiesel production (Mata *et al.* 2010; Lv *et al.* 2010). It is not surprising that many researchers try to increase its oil-productivity with different approaches (Al-Iwayzy *et al.* 2014; Belotti *et al.* 2013; Praveenkumar *et al.* 2012; Mallick *et al.* 2012; Mujtaba *et al.* 2012; Kurt 2011; Yeh and Chang 2011; Guarnieri *et al.* 2011; Scott *et al.* 2010; Converti *et al.* 2009; Liu *et al.* 2008; Illman *et al.* 2000; Miyachi *et al.* 1978; Harris *et al.* 1965).

2.4.1. Increase of Oil-Production by Altering Culture and Stress Conditions

To increase the oil-productivity of *C. vulgaris*, different approaches can be applied, such as the changing from phototrophic to hetero- or mixotrophic conditions or by altering of abiotic (temperature, pH) or biotic factors (i. e. nutrient limitation) to stress the cells. Also metabolic genetic engineering plays an important role and were already applied to the model *C. reinhardtii* (La Russa *et al.* 2012; Merchant *et al.* 2012), but have not been reported for *Chlorella,* yet. Different approaches will be reviewed in which the oil-content of *C. vulgaris* was significantly increased by altering its culture conditions. This also is an important tool to identify potential bottlenecks for a subsequent genetic engineering project.

The changing of the growing conditions from photo- to mixo- or heterotrophic has been shown to dramatically change the compound composition of *C. vulgaris*. According to Spoehr and Milner (1948), the proportions can vary between 58 % protein and 4.5 % lipid to 8.7 % protein and 86 % lipid. Xu *et al.* (2006) demonstrated an increase of the lipid fraction from 14.6 % to 55.2 % by switching from phototrophic to heterotrophic conditions. Further publications demonstrate also the success in increased oil-content when changing the growing conditions to hetero- or mixotrophic (Belotti *et al.* 2013; Mallick *et al.* 2012; Liang *et al.* 2009).

Several reports showed the impacts of environmental alterations on the lipid-productivity and FAME composition of *C. vulgaris* (Table 2.1). In many microalgae exposure to stress results in dramatic changes in metabolism towards the formation of neutral lipids. Therefore, a focus especially on non-polar lipid or TAG accumulation was determined (Merchant *et al.* 2012; Hu *et al.* 2008). For example, a rise of the culture-temperature from 25 °C to 30 °C led to an ~ 2.5-fold decrease of the lipid productivity (Converti *et al.* 2009). Also the wavelength of light showed a high impact on the *C. vulgaris* composition. While, red light resulted in a greater incorporation of labeled $^{14}CO_2$ into sucrose and starch, blue light led to a preferred incorporation into i. a. the lipid fraction (Miyachi *et al.* 1978). A number of investigations were made on *C. vulgaris* when cultured in nitrogen-depleted conditions, as the lipid content has been found to increase by up to 60 % DCW and a productivity of up to 78 mg/L/d under these conditions (Table 2.1). Additionally, under nitrogen depletion *C. vulgaris* showed an altered FAME profile with improved properties for biodiesel containing mainly saturated C16 and C18 FAs and monounsaturated FAs up to 90 % of total lipids (Mallick *et al.* 2012; Praveenkumar *et al.* 2012; Guarnieri *et al.* 2011; Yeh and Chang 2011; Kurt 2011; Scott *et al.* 2010; Illman *et al.* 2000; Harris *et al.* 1965). When *C. vulgaris* cultures in the late exponential phase were supplemented with additional iron (Fe^{3+}) this resulted in a high increase of the oil-content up to 56.6 % DCW (Liu *et al.* 2008). Whereas iron deprivation resulted in no significant increase in oil-production (Praveenkumar *et al.* 2012). Potassium-phosphate deprivation led also not to a significant increase of the lipid content in *C. vulgaris*, but it resulted also in a high increase of saturated FAs (C16 and C18) and monounsaturated FAs with 83.8 % (Praveenkumar *et al.* 2012). Combined deprivations of nutrients resulted in improvements of the TAG yield up to 53 % and a lipid productivity of up to 77.1 mg/L/d (Mujtaba *et al.* 2012; Praveenkumar *et al.* 2012). Interestingly, it has been found that the CO_2 incorporation into the lipid fraction was highly increased when cells were nutrient starved for 24 hours. Before nutrient depletion 28 % of the total supplied CO_2 were incorporated into lipids, after that time the incorporation of CO_2 were up-regulated to 58 % (Miyachi *et al.* 1978).

The growing conditions (photo-, mixo- or heterotropic) and the induced nutrient stress can successfully be combined, resulting in a high lipid content and high TAG productivity rate. For example, Belotti *et al.* (2013) combined the alteration of growing conditions with deprivations and limitations of nitrogen and phosphorus. They detected the highest lipid productivity when cells grew under mixotrophic conditions with a lack of phosphorus and a restricted availability of nitrogen. Under those conditions the total lipid productivity of *C. vulgaris* was determined to reach 135 mg/L/d. In comparison to the growth under phototrophic and heterotrophic conditions with the same limitations and deprivations only 39 mg/L/d and 65 mg/L/d were reached, respectively (Belotti *et al.* 2013).

Table 2.1 | Summary of impacts of environmental factors on the lipid productivity and FAME content determined for *C. vulgaris*.

Factor	Condition	Observed biochemical alteration	Reference
Temperature	rising	the change from 25 °C to 30 °C for culturing decreased the lipid content from 14.7 % to 5.9 % DCW, respectively	Converti *et al.* 2009
Light	blue light	monochromatic blue light enhanced the CO_2 incorporation into lipids	Miyachi *et al.* 1978
Nitrogen	low nitrogen	cultures were grown with media containing low, media and high concentrations of nitrogen; the total extracted FAME content was determined to be 40 %, 32.6 % and 13 %, respectively	Kurt 2011
	low nitrogen	0.313 g/L KNO_3 led to a lipid content of 55.9 % and a productivity of 78 mg/L/d	Yeh and Chang 2011
	low nitrogen	resulted not only in enhanced lipid accumulation but also in an altered lipid composition form total oil extracted; 66.6 % of total lipids were determined to be free FA (C16 and C18) when cultured under normal conditions (TAGs: 5.7 %), whereas mostly TAGs are present after 17 days of nitrogen depletion with 74.2 % of total lipids (free FA: 15.1 %)	Widjaja *et al.* 2009
	low nitrogen	increased oil-productivity from 18 % to 40 % and a deceased protein level from 29 % to 7 % DCW	Illman *et al.* 2000
	deprivation	lipid content of the cell increased from 31 % to 43 % DCW with a FAME profile of 76 % saturated and monounsaturated FA of total lipids	Praveenkumar *et al.* 2012
	deprivation	C18:1 content increases significantly followed by C18:0 and C16:0	Harris *et al.* 1965
	replete / deplete	under nutrient replete conditions accumulation of 10 % DCW were determined, when grown for 5 days in the absence of nitrogen in the media the oil-content reached 60 % DCW of total lipids	Guarnieri *et al.* 2011
	reduction	the reduction of $NaNO_3$ concentration form 1.5 g/L to 0.75 g/L and 0.375 g/L in the growth media resulted in an increase of the lipid content form 5.9 % to 14.4 %, and 15.3 % DCW, respectively	Converti *et al.* 2009
Iron	high concentration	optimal conditions increased total lipid content of up to 56.6% biomass by dry weight	Liu *et al.* 2008
	supplied	increased lipid content	Al-Iwayzy *et al.* 2014
	deprivation	lipid content of the cell increased slightly from 31.2 % to 31.4 % DCW with a FAME profile of 78 % saturated and monounsaturated FA of total lipids	Praveenkumar *et al.* 2012
Phosphate	deprivation	lipid content of the cell increased showed no significant increase (31 % to 32 % DCW); the FAME profile exhibits 78 % saturated and monounsaturated FA of total lipids	Praveenkumar *et al.* 2012
Nutrient combinations	containing a general starvation step	53 % DCW lipids consisted of lipids after 24 hours at an optimal 2nd stage conditions containing nitrogen deprivation	Mujtaba *et al.* 2012
	nitrate, iron, and phosphate deprivation	lipid content of the cell increased from 31 % to 41 % DCW with a FAME profile of 65 % saturated and monounsaturated FA of total lipids	Praveenkumar *et al.* 2012
	general starvation	CO_2 incorporation into the lipid fraction was highly increased when cells were nutrient starved for 24 hours before measurement; 28 % incorporation before and 58 % CO_2 incorporation of total supplied CO_2 after starvation were determined	Miyachi *et al.* 1978

2.5. *TAG-Biosynthesis Pathway in Plants and Microalgae*

Triacylglycerols (TAGs) belong to the energy richest carbon forms found in nature and are used in various organisms as energy storage compounds. TAGs, which were also described as neutral or non-polar lipids, consists of three FA chains with a length of 16 to 18 carbons esterified via the hydroxyl groups to one molecule glycerol (Durrett *et al.* 2008). In the past, researcher assumed the TAG-biosynthesis of microalgae is highly similar to those occurring in plants (Harris *et al.* 1965), but recent studies showed that the pathways differ in several aspects (Liu and Benning 2013; Merchant *et al.* 2012). TAG-biosynthesis of the free FAs and TAGs occurs in chloroplasts and endoplasmic reticulum (ER) of microalgae cells, respectively (Liu and Benning 2013; Yu *et al.* 2011).

A simplified hypothetical overview of TAG-biosynthesis in microalgae is shown in Figure 2.3.

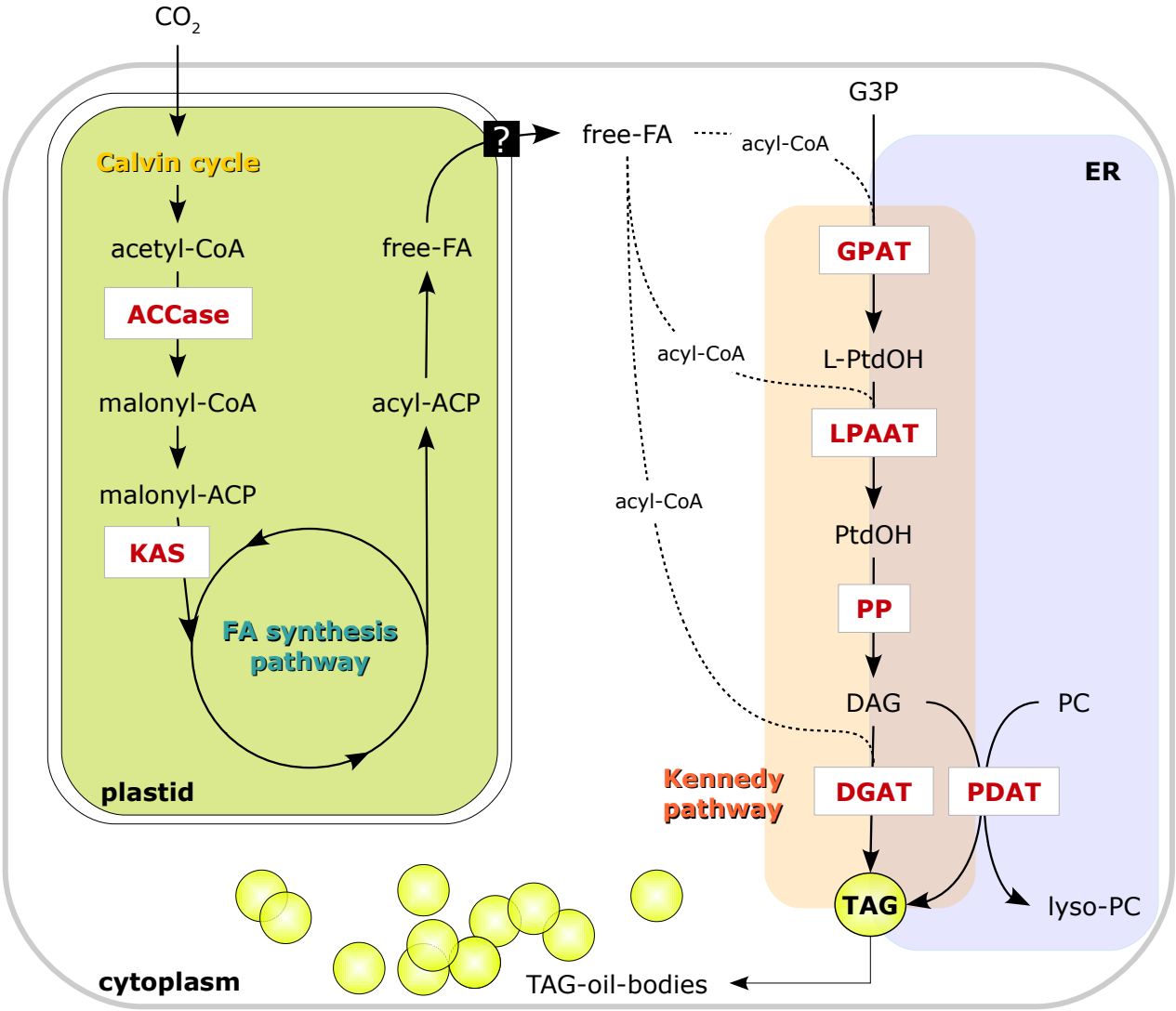

Figure 2.3 | Simplified hypothetical overview of the TAG-biosynthetic pathway in microalgae. Abbreviations: **ACCase**: acetyl-CoA-carboxylase; **ACP**: acyl carrier protein; **CoA**: coenzyme A; **DAG**: diacylglycerol; **DGAT**: diacylglycerol acyltransferase; **ER**: endoplasmic reticulum; **FA:** fatty acid; **G3P**: glycerol-3-phosphate; **GPAT**: sn-glycerol-3-phosphate acyltransferase; **KAS**: 3-ketoacyl-acyl-carrier protein synthase; **L-PtdOH**: Lyso-phosphatidic acid; **lyso-PC**: lyso-phosphatidylcholines; **LPAAT**: lyso-phosphatidic acid acyltransferase; **PC**: phosphatidylcholines; **PDAT**: phospholipid diacylglycerol acyltransferase; **PP**: phosphatidic acid phosphatase; **PtdOH**: phosphatidic acid; **TAG**: triacylglycerol.

In the chloroplasts the synthesis of free FAs were processed in three steps. In the first committed step of the FA pathway the enzyme acyl-CoA-carboxylase (ACCase) catalyzes the irreversible carboxylation of acetyl-CoA to malonyl-CoA. After the transfer to an acyl carrier protein (ACP), malonyl-ACP enters the FA pathway during another conversion catalyzed by 3-ketoacyl-ACP synthase (KAS). In the FA pathway, malonyl-ACP condenses with another acyl-ACP and were elongated through the action of several enzymes of the FA complex. In each cycle, two carbons are added to the ACP-bound FA chain, eventually forming C16:0-ACP. In the final step, C16:0-ACP can elongate and undergo desaturation to form C18:0-ACP and C18:1-ACP, respectively (Lung and Weselake 2006). Those FAs exit the chloroplast and enter the cytosol in an esterified form known as acyl-CoA.

Acyl-CoA are transferred to the ER membrane where the TAG-biosynthesis pathway is located, also called *Kennedy pathway* (Kennedy 1961). To generate TAG, the acyl-CoA chains are attached in several steps to one molecule glycerol, which is derived from glycerol-3-phosphate (G3P). This molecule develops from the conversion of dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase (G3PDH), an important enzyme to provide the backbone of the TAG molecule. In the *Kennedy pathway* acyltransferases sequentially attach in total three FA chains to glycerol to form TAG (Figure 2.4). Therefore, *sn*-glycerol-3-phosphate acyltransferase (GPAT), lyso-phosphatidic acid acyltransferase (LPAAT), and diacylglycerol acyltransferase (DGAT) are the key

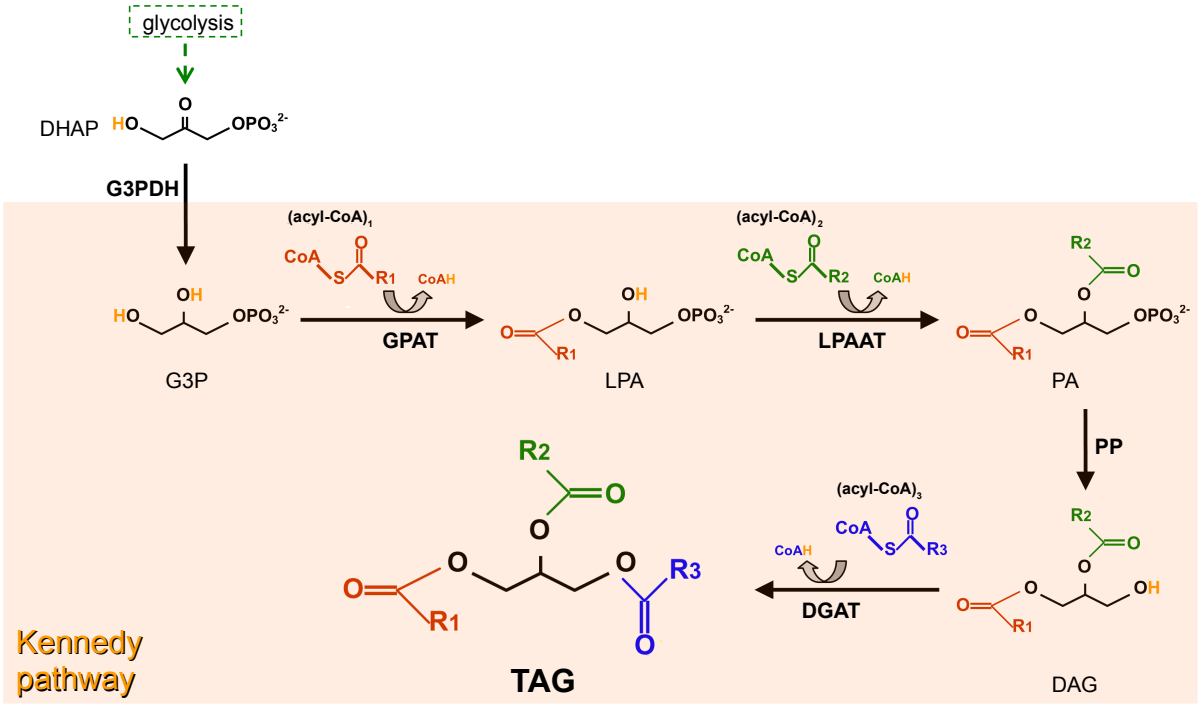

Figure 2.4 | Overview of the TAG-biosynthesis reactions of the *Kennedy pathway*. G3PDH catalyzes a reaction to provide the glycerol backbone for TAG-production. In the *Kennedy pathway* (pink background) three acyl transferases (GPAT, LPAAT and DGAT) attach acyl-CoAs or FA chains to the glycerol molecule to form TAG. Abbreviations: **CoA**: coenzyme A; **DAG**: diacylglycerol; **DGAT**: diacylglycerol acyltransferase; **DHAP**: dihydroxyacetone phosphate; **G3P**: glycerol-3-phosphate; **GPAT**: *sn*-glycerol-3-phosphate acyltransferase; **LPA**: lysophosphatidic acid **LPAAT**: lyso-phosphatidic acid acyltransferase; PA: phosphatidate; **PP**: phosphatidic acid phosphatase; **TAG**: triacylglycerol. **R1**, **R2** and **R3** represent acyl groups of the FA chain. Modified from Kurt 2011.

enzymes for TAG-production (Radakovits *et al.* 2010; Yu *et al.* 2011), whereas DGAT catalyzes the last reaction to form TAG (Guihéneuf *et al.* 2011). Finally, the TAG molecules accumulate within the ER membrane in lipid bodies, become coated with oleosin, and were released into the cytoplasm (Lung and Weselake 2006). A major contributor to TAG synthesis is phospholipid:diacylglycerol acyltransferase (PDAT). This enzyme acts acyl-CoA independent and catalyzes the transfer of the third acyl-unit from phosphatidylcholines (PC; instead of acyl-CoA) to diacylglycerol (DAG) to form TAGs (Boyle *et al.* 2012; Lung and Weselake 2006; Dahlqvist *et al.* 2000).

2.6. Identification of Potential Bottlenecks for TAG-Production

To identify and characterize potential bottlenecks for TAG-production, several attempts to increase oil-content through metabolic engineering were performed for plants and microalgae (Yu *et al.* 2011). As the plant TAG-biosynthesis pathway is the most similar one to microalgae and well-studied, and because only a few reports about genetic engineering of microalgae for increased oil-production were published, we mainly considered reports on changes in the TAG-metabolism in plants triggered by genetic engineering of involved enzymes in this section.

Several genes involved in the FA pathway have been suspected to act as potential bottlenecks for TAG-production and were characterized by overexpression. Those investigations resulted all in a small or even no increase in TAG levels. For instance, Roesler *et al.* (1997) overexpressed a cytosolic version of ACCase, which was targeted to the chloroplast in rapeseed. Typically, ACCase activity is considered to be low in mature *B. napus* seeds showed a 10 – 20-fold increase in expression level in the overexpressing lines. This resulted in an altered seed FA composition with a highly increased oleic acid content, but also in an increase in oil up to 5 % (Roesler *et al.* 1997). For microalgae, Dunahay *et al.* (1996) performed an experiment by overexpressing ACCase from the diatom *Cyclotella cryptica,* resulting in a 2 – 3-fold increase in ACCase levels, but no detectable increase in TAG content (Dunahay *et al.* 1996). The overexpression of spinach KAS (type III; Figure 2.3), resulted in an increased C16 saturated FAs level in tobacco and rapeseed. The up-regulation of palmitic acid was determined to 30 % and 100 % for tobacco and rapeseed, respectively. The KAS enzyme activity of transformed tobacco was 300-fold up-regulated, but no significant increase in oil-productivity was determined (Dehesh *et al.* 2001). Blatti *et al.* (2013) reviewed recent attempts to engineer microalgae with an improved TAG level by altering the FA pathway through overexpression of its enzymes (ACCase, KAS and thioesterase). This review confirms the findings described above and proved thereby that also in microalgae the overexpression of enzymes of the FA pathway do not result in an increased TAG level (Blatti *et al.* 2013). These results led us to conclude that the main enzymes of the FA pathway, such as ACCase and KAS, just play a minor roles in limiting TAG-production and are not the primary bottlenecks in oil-biosynthesis.

In contrast, overexpression of enzymes of the *Kennedy pathway* resulted in a significantly higher TAG levels, as several studies reported an increase up to 40 % (Vigeolas *et al.* 2007; Zou *et al.* 1997). For example, the overexpression of yeast G3PDH in rapeseed, which leads to the formation of G3P,

entering the *Kennedy pathway* and provides the glycerol backbone of the TAG molecules (Figure 2.3 and 2.4; Vigeolas *et al.* 2007). They found a two-fold increase in G3PDH activity in developing seeds resulting in a 40 % increase of the total lipids in the transgenic seeds (Vigeolas *et al.* 2007). Overexpression studies of GPAT, catalyzing the first reaction in the *Kennedy pathway* (Figure 2.3 and 2.4), resulted in an increase of oil up to 29 % (22 % in average form three transgenic lines; Jain *et al.* 2000). Also the second acyltransferase in the pathway, LPAAT (Figure 2.3 and 2.4), resulted in an increased oil-content. Therefore, Zou *et al.* (1997) overexpressed the yeast *lpaat* gene SLC1-1 in *Arabidopsis thaliana* and *B. napus*, also resulting in an increased seed oil-content of 8 - 40 % (Zou *et al.* 1997). Diacylglycerol acyltransferase, catalyzing the last step in the *Kennedy pathway* (Figure 2.3 and 2.4), were also determined in several studies to increase the total lipid content. The expression of DGAT in *Nicotiana tabacum* led to in 20-fold increase of TAG accumulation compared to the unmodified control plants and to an overall two-fold increase in extractable FAs (Andrianov *et al.* 2010). In *A. thaliana* the recombinant expression of DGAT resulted in a 5.9-fold increase in oil-content compared to the positive control (Petrie *et al.* 2012) and the codon-optimized version of DGAT from soil fungus *Umbelopsis ramanniana* expressed in soybean seeds resulted in an absolute increase 1.5 % oil by weight (Lardizabal *et al.* 2008).

These findings lead to the assumption that the bottlenecks for TAG-production are rather to be found in the *Kennedy pathway* than in the FA pathway for both plants and microalgae. This has been confirmed by transcriptom investigations performed for *C. vulgaris*. The expression levels of enzymes from the FA and *Kennedy pathway* were investigated and compared when grown under nitrogen enriched and nitrogen depleted conditions. It has been determined that enzymes of the FA pathway are up-regulated just up to two-fold (ACCase and KAS) or even down-regulated. In contrast, enzymes of the *Kennedy pathway* were found to be up-regulated from 18-fold up to 133-fold. The greatest increase of transcription level was observed for the DGAT enzyme (Guarnieri *et al.* 2011).

Currently, the enzyme DGAT is discussed to be one of the most limiting enzymes for TAG-production. Since then, several laboratories work on the overcoming of this bottleneck by characterization and attempts of genetic engineering in microalgae to increase oil-productivity (Gong *et al.* 2013; La Russa *et al.* 2012; Guihéneuf *et al.* 2011; Wagner *et al.* 2010). The DGAT1 enzyme will be further discussed in the following section (Section 2.7).

2.7. Diacylglycerol Acyltransferase Type-1 (DGAT1)

In the *Kennedy pathway* diacylglycerol acyltransferase (DGAT) is the enzyme, which catalyzes the last step in TAG-biosynthesis before lipid droplets are formed and released to the cytosol (Figure 2.5; Kennedy 1961). Three types of DGAT have been identified, DGAT1 to DGAT3, and all seem to play a role in TAG-production. The DGAT types 1 and 2 are located in the ER membrane and play the major role in oil synthesis (Guihéneuf *et al.* 2011). Both have been found in several eukaryotic organisms, including humans, vertebrates, higher plants, but also in fungi, lower animals and microalgae (source: DGAT1 global protein search at *National Center for Biotechnology Information*; NCBI). Even when

they catalyze the same reaction, DGAT1 and DGAT2 do not show sequence similarities (Chen and Smith 2012; La Russa *et al.* 2012; Lung and Weselake 2006). DGAT3 is a soluble cytoplasmic enzyme, which exact function in TAG synthesis is not known yet (Guihéneuf *et al.* 2011; Saha *et al.* 2006), but it seems to play a minor role in TAG-production (Lung and Weselake 2006).

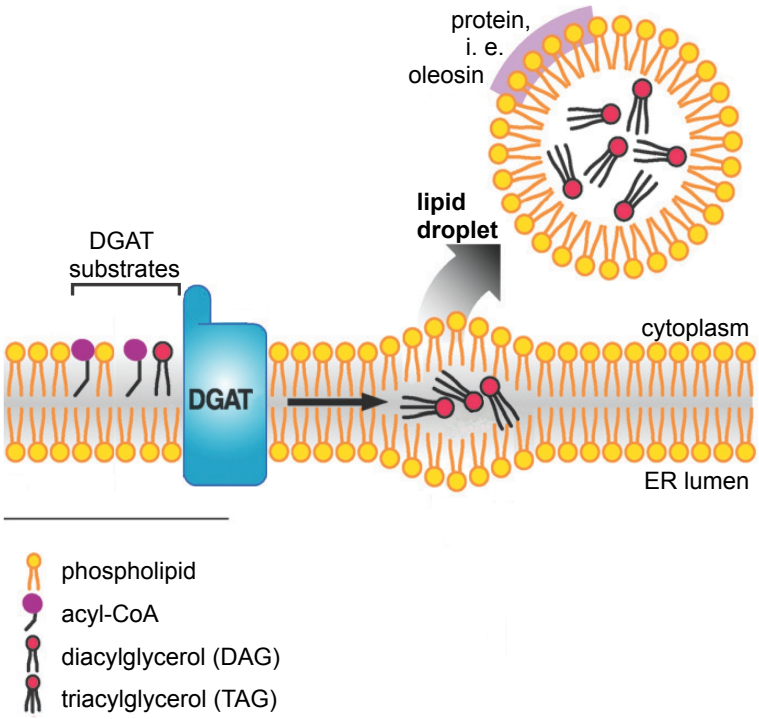

Figure 2.5 | Hypothetical model illustrating the last catalyzed step in TAG-biosynthesis (*Kennedy pathway*) by the enzyme DGAT localized within the ER membrane. TAG is assumed to be channeled into the cores of cytosolic lipid droplets; modified from Yen *et al.* 2008.

In several plant and microalgal studies the DGAT enzyme has been identified to be one of the key and rate-limiting enzymes in TAG-production (Li *et al.* 2013; Guarnieri *et al.* 2011; Miller *et al.* 2010; Lung and Weselake 2006; Jako *et al.* 2001; Perry *et al.* 1999; Zou *et al.* 1999; Ohlrogge and Jaworski 1997; Ichihara *et al.* 1988). Even when DGAT1 and DGAT2 isoenzymes have been demonstrated to significantly increase TAG-production (i. e. yeast mutants) it has also been shown that the lipid metabolism across algae and plants are different regarding to the type of DGAT they prefer (Liu and Benning 2013). The preference of the use of DGAT1 or DGAT2 seem to be highly tissue- and species-dependent. Although DGAT1 plays a major role in some plants, the DGAT2 enzyme can be the dominant catalyzing enzyme in other plants (Shockey *et al.* 2006).

DGAT1 is a protein of the membrane bound O-acyltransferase (MBOAT) superfamily. It is embedded into the ER membrane with eight to ten hydrophobic transmembrane domains. All members of the MBOAT superfamily transfer organic acids, mostly FAs, onto hydroxyl groups of membrane-embedded targets, such as the DGAT1 protein (Hofmann 2000). Based on the sequence homologies with mammalian acetyl-CoA acetyltransferase (ACAT), the first cloned DGAT1 was isolated from mouse

and characterized in H5 insect cells by Cases and coworkers (1998). In plants, DGAT1 was first described in *Arabidopsis thaliana* (Zou *et al.* 1999) followed by various numbers of other DGAT1 (McFie *et al.* 2010; Mañas-Fernández *et al.* 2009; Shockey *et al.* 2006; Ohlrogge and Jaworski 1997). It was found, that DGAT1 from different organisms share distinctive motifs of functional importance, with most of them being highly conserved, these include the binding motifs for the two substrates, acyl-CoA and diacylglycerol (DAG), the putative active site, but also the fatty acid signature motif (FYxDWWN), and a dominant phenylalanine, which demonstrated a high impact on enzyme activity (Guihéneuf *et al.* 2011; Turchetto-Zolet *et al.* 2011; Mañas-Fernández *et al.* 2009; Zheng *et al.* 2008; Xu *et al.* 2008; Coleman and Lee 2004; Jako *et al.* 2001; Guo *et al.* 2001; Oelkers *et al.* 1998).

The DGAT1 enzyme has been demonstrated to play the major role in TAG biosynthesis not only in *C. reinhardtii*, but also in *C. vulgaris*. For example, Miller *et al.* (2010) investigated major changes in mRNA levels following nitrogen deprivation in *C. reinhardtii*. They found that DGAT1 is almost completely suppressed under nitrogen abundant conditions. In contrary, the expression levels following nitrogen deprivation showed a large increase in transcript abundance of DGAT1 (Miller *et al.* 2010). This result has also been reported by Guarnieri *et al.* (2011). They investigated the transcriptome of *C. vulgaris* under nitrogen depletion, when high lipid accumulation occurred. Guarnieri *et al.* (2011) found that the expression of all proteins of the *Kennedy pathway* were highly increased from 16.6 to 133.6-fold; in particular, DGAT1 showed the highest rise in expression based on sequence homologies.

2.8. Genetic Engineering of Microalgae

Genetic engineering of microalgae and its scalable cultivation in bioreactors will favor its applicability as cell-factories for producing new bioactive products (i. e. high-quality hormones and antibodies) or enhancing the productivity of naturally occurring compounds (León-Bañares 2004; Zaslavskaia *et al.* 2001).

Unfortunately, the genetic engineering of microalgae is still challenging. The main problem is the delivery and the stable integration of foreign DNA into the algal genome (León and Fernández 2007; León-Bañares 2004). The very tough outer cell wall acts as a physical barrier to DNA delivery (Radakovits *et al.* 2010; Huang *et al.* 2006). Successful transformation seems to be species-dependent, requiring optimized protocols for each microalgae species (Radakovits *et al.* 2010). A further problem for foreign gene expression is the biased codon usage of microalgae, which restricts its expression efficiency. This makes codon usage adaptation for microalgae inevitable (León and Fernández 2007; León-Bañares 2004; Zaslavskaia, *et al.* 2000; Mayfield and Kindle 1990). As mentioned above, in green algae a high GC-content is frequently found (Jarvis *et al.* 1992; Mayfield and Kindle 1990; Kessler 1976). In *C. reinhardtii*, the high GC-content is caused by the exclusion of adenine in the third codon position, except for the stop codon (Mayfield and Kindle 1990). The high GC-content in turn might result in a deviation of the transcriptional and translational regulatory mechanisms found in other organisms, like the recognition of the promoter region, transcription

termination (polyadenylation), RNA instability, ribosome-binding, or gene-silencing caused by methylation or epigenetic effects (León and Fernández 2007; León-Bañares 2004; Mayfield and Kindle 1990). These characteristics of microalgae hamper the applicability of microalgae as cell-factories for foreign gene expression.

To circumvent these limitations, homologous genes are applied in enabling efficient transformation (Kindle 1990; Mayfield and Kindle 1990). Recently, an increasing number of working groups focus on the overexpression of naturally occurring genes, which are assumed to be pathway bottlenecks, to increase the productivity of naturally occurring algae metabolites, such as carotenoids, polyunsaturated fatty acids, hydrogen or TAGs (Trentacoste *et al.* 2013; La Russa *et al.* 2012; Moellering and Benning 2010; Del Campo *et al.* 2007; Steinbrenner and Sandmann 2006; Dunahay *et al.* 1996). Our research is focused on developing a comprehensive engineering protocol to enable the investigation of biosynthetic enzymes involved in oil-biosynthesis of *C. vulgaris*.

2.8.1. What to Consider for Plasmid Construction

The genetic engineering of microalgae requires a suitable vector system and a highly-efficient expression cassette. The expression cassette should include a suitable selection system, and a strong promoter.

For the efficient selection of transformants, a suitable **selection system** has to be determined as some microalgae species are resistant to specific antibiotics. For example, kanamycin and chloramphenicol are used for selection of *Chlamydomonas* species, but not for *Chlorella* species (Chow and Tung 1999). Additionally, *C. vulgaris* is insensitive to ampicillin and streptomycin (Hawkins and Nakamura 1999). Another consideration is the influence of the media composition on the effect of the antibiotic. For example, the antibiotic gentamicin (G418) is ineffective in diatoms or green algae species cultivated under a high salt concentrations but effective at low salt concentrations (Zaslavskaia, *et al.* 2000). In *Chlamydomonas*, successful selection markers include paromomycin, emetine, and hygromycin B (hygB; Neupert *et al.* 2012). In the fresh water *Chlorella* species, the antibiotics hygB (Chow and Tung 1999; Huang *et al.* 2006), G418 (Chen *et al.* 2001; Hawkins and Nakamura 1999), and zeomycin (Strenkert *et al.* 2013; Lumbreras *et al.* 1998) are commonly used for selection.

For the choice of the **vector**, size plays a decisive role. When the vector size is too small, it becomes less stable in *Agrobacterium*, when used *Agrobacterium*-mediated transformation (Murai 2013), but when the vector size is too big (> 15 kbp), the isolation and handling of the plasmid is difficult, especially for insertion of recombinant genes, which is generally known. Commonly used plasmids for genetic engineering of microalgae are between 9.5 kbp and 16.1 kbp (La Russa *et al.* 2012; Huang *et al.* 2006; Kumar *et al.* 2004; Hawkins and Nakamura 1999; Lumbreras *et al.* 1998; Chen *et al.* 2001; Chow and Tung 1999; Table 2.2). Preferably, the vector should contain the desired selection cassette,

a multiple cloning site for inserting the expression cassette directly in the vector, and an efficient replicon for a high copy number in *E. coli* for enrichment of the desired plasmids.

The pMDC162 binary vector contains the hygB resistance gene as selection cassette within the T_i-region and contains additional features, which make it an attractive vector for genetic engineering of microalgae. The pMDC162 has a size of 12,886 bp and is derived from the pCAMBIA T-DNA cloning vectors (Curtis and Grossniklaus 2003). pCAMBIA vectors are verified comprehensively in a range of plant species[1] and microalgae. For instance, the transformation of *C. reinhardtii* with the vector pCAMBIA1304 resulted in successful expression of the *gus* and the green fluorescent protein (*gfp)* reporter gene (Kumar *et al.* 2004; Table 2.2).

Table 2.2 | Comparison of different binary vector sizes used for transformation of microalgae species.

Binary vector system	Vector size	Organism used for	Reference
pCAMBIA1304	~ 11.8 kbp *	*Chlamydomonas reinhardtii*	Kumar *et al.* 2004
pPZP111	9.5 – 11.0 kbp *	*Chlamydomonas reinhardtii*	Hawkins and Nakamura 1999
pSP108	12.0 kbp *	*Chlamydomonas reinhardtii*	Lumbreras *et al.* 1998
pHm3A	~ 13.0 kbp	*Chlorella sp. DT*	Huang *et al.* 2006
pBin19	~ 15.2 – 16.1 kbp *	*Chlorella ellipsoidea*	Chen *et al.* 2001
pIG121-Hm	~ 15.0 kbp *	*Chlorella vulgaris*	Chow and Tung 1999
pMDC162	11.3 – 12.1 kbp	*Chlorella vulgaris*	this study

* Estimations of vector sizes provided by snappgene.com and not provided in the paper. The total vector size was estimated by summing the gene size from National Center for Biotechnology Information (NCBI) and the vector size.

The choice of a **promoter** is a crucial step when designing a high producing expression cassette in general (Vila *et al.* 2012; León and Fernández 2007; Schroda *et al.* 2000). In *C. reinhardtii*, most endogenous and foreign promoters resulted in poor expression levels. In contrast, the choice of a terminator is less problematic (Neupert *et al.* 2012).

Promoters can be distinguished in constitutive and inducible promoters. Constitutive promoters drive gene expression continuously in a well-detectable level, but might further be up- or down-regulated by different stimuli (Gurr and Rushton 2005). Examples are the 35S promoter derived from the Cauliflower Mosaic Virus (CaMV 35S), the mannopine synthase promoter (MAS) or the polyubiquitin-1 promoter (Ubi1). In contrast, inducible promoters lack basal expression or the expression level is very low, but they are highly activated or further inducible through one or more stimuli. Those activators can be hormones (auxin, cytokinin, methyl jasmonate, ethylene), environmental conditions (light, temperature), or stress (drought/salt stress, wounding). Typical examples are the promoter of the

1 http://www.cambia.org/daisy/cambia/585.html#dsy585_References; status Sept. 29th 2014

small subunit 2 of ribulose-1,5-bisphosphate carboxylase / oxygenase (RBCS2) and the heat shock protein 70A promoter (Hsp70A; Gurr and Rushton 2005). It is desirable not only to use constitutive promoters, but also inducible promoters, or constitutive promoters, which are further inducible. For instance, the microalgae can first be grown up to a moderate density before expression is turned on. This is an important tool, especially, when the gene product is toxic to the host or affects the cell growth.

For the efficient genetic engineering of microalgae, the most commonly used and well-studied promoter fusion for *C. reinhardtii* is the Hsp70A/RBCS2 chimeric promoter system (Schroda *et al.* 2000; Strenkert *et al.* 2013; Eichler-Stahlberg *et al.* 2009; Li and Tsai 2009; Chen *et al.* 2008). The RBCS2 promoter and the Hsp70A promoter alone were also found in several applications (Kim *et al.* 2002; Schroda *et al.* 2000; Hawkins and Nakamura 1999; Lumbreras *et al.* 1998). Another strong promoter used in microalgae is the constitutive PsaD promoter. It was derived from the photosystem I protein subunit and proved to drive high expression in *C. reinhardtii* (La Russa *et al.* 2012; Croft *et al.* 2007), as well as the Ubi1 promoter (Chen *et al.* 2001; Taylor *et al.* 1993). The CaMV 35S was used for the first attempts of engineering microalgae but lost its relevance due to its varying success and lower expression level compared to other developed promoters or promoter systems (Huang *et al.* 2006; Kumar *et al.* 2004; Kim *et al.* 2002; Chen *et al.* 2001; Hawkins and Nakamura 1999; Chow and Tung 1999; Jarvis and Brown 1991).

The **reporter** genes luciferase (*luc*), the green and yellow fluorescent protein gene (*gfp* and *yfp*) and the ß-glucuronidase gene (*gus*), which are routinely applied in plants, are widely used for algae as well. Jarvis and Brown (1991) were the first successfully expressed the *luc* gene in *Chlorella ellipsoidea* protoplasts (Jarvis and Brown 1991). Nowadays, *luc* is rarely used as a reporter gene for microalgae. The *gfp* and *yfp* genes were applied in *C. ellipsoidea* (Kim *et al.* 2002) and in *C. reinhardtii* (Kumar *et al.* 2004; Neupert *et al.* 2012), but were not an efficient reporter for *C. reinhardtii*, unless adjusted strains were used (Neupert *et al.* 2012). The *gus* reporter gene was first successfully expressed in *Chlorella saccharophila* cells by electroporation (Maruyama *et al.* 1994). Since then, the *gus* reporter gene has been found commonly applied in microalgae, for example in *C. vulgaris* (Chow and Tung 1999), *C. ellipsoidea* (Chen *et al.* 2001), *C. reinhardtii* (Kumar *et al.* 2004) and *Chlorella* sp. DT (Huang *et al.* 2006).

In summary, in this section it is described that several important elements has to be well-considered before starting the assembling of the final expression cassette. As the right choice of a selection system, vector, reporter gene and promoter will decide about the success of the subsequent genetic engineering experiment.

2.8.2. Transformation of Microalgae

The most crucial step for engineering microalgae is the transformation and the stable integration into the algal genome. For every microalgae species, the transformation has to be optimized and the transformation efficiency is strongly species-dependent (Radakovits *et al.* 2010; Hallmann 2007). The transformation efficiency and the number of stable transformants are believed to be inversely correlated with size and the complexity of the alga (Hallmann 2007). Only a few species are routinely transformed, these include *C. reinhardtii*, *Volvox carteri*, the diatom *Phaeodactylum tricornutum*, and several *Chlorella* species (Walker *et al.* 2005; León-Bañares 2004). Recently, an additional transformation protocol for the nuclear DNA-insertion in *Nannochloropsis* has been published (Radakovits *et al.* 2012).

A variety of methods have been used to transform microalgae. These include the glass-bead method (Kindle 1990), the PEG method, which were derived from the glass-bead method (Jarvis and Brown 1991), the silicon carbide (SiC) method (Dunahay 1993), electroporation (Maruyama *et al.* 1994), biolistic transfection or particle bombardment (Mayfield and Kindle 1990) and the *Agrobacterium*-mediated transformation method (Kumar *et al.* 2004).

The **glass-bead** method is a cheap and simple transformation procedure. However, it is recommended for cell-wall-deficient strains (available for *C. reinhardtii*) or for protoplasts to obtain a high transformation efficiency. DNA-integration occurred when protoplasts were agitated in the presence of polyethylene glycol (PEG), glass-beads (0.4 – 0.5 mm in diameter) and DNA (Kindle 1990). This glass-bead method has been demonstrated in *C. ellipsoidea* without agitation (Jarvis and Brown 1991). This simplified **PEG-method** was shown to work in several other *Chlorella* species, including *C. ellipsoidea* (Kim *et al.* 2002), *Chlorella* sp. DT (Huang *et al.* 2006), *C. sorokiniana* and *C. vulgaris* (Hawkins and Nakamura 1999).

The **SiC-method** is similar to the glass-bead and PEG method and was developed for *C. reinhardtii* by Dunahay in 1993. The main difference between the SiC-method and other methods is that cells with intact cell walls can be transformed. This method applies silicon carbide nano-particles instead of glass-beads to intact cells agitating in the presence of PEG (Dunahay 1993), (León and Fernández 2007; Hallmann 2007). In addition to *C. reinhardtii*, this method was demonstrated in dinoflagellates (ten Lohuis and Miller 1998).

Electroporation is a method in which cells with thin walls, protoplasts, or cell-wall-deficient mutants can easily be transformed. For electroporation, a suspension of cells and DNA is placed in a special cuvette with two electrodes and placed in an electroporator. Here, a voltage is applied, which exceeds the dielectric strength of the plasma membrane during the electric pulse. When the phospholipid bilayer of the cells is temporarily disrupted, DNA molecules pass through the cell membranes (Hallmann 2007). Electroporation is a straight-forward method, as the cells and protoplasts do not need special treatments. Unfortunately, stable integration into the genome is rare (Chow and Tung 1999). Electroporation was performed successfully in *C. reinhardtii* (Shimogawara *et al.* 1998), but also in *C. saccharophila* (Maruyama *et al.* 1994), *Chlorella* sp. DT (Huang *et al.* 2006), *C. ellipsoidea* (Chen *et al.* 2001), and *C. vulgaris* (Chow and Tung 1999).

The **particle bombardment** or biolistic transfection method bombards target tissue or cells with DNA-coated nano-particles using a *gene-gun*. Small gold particles (0.5 – 1.5 µm in diameter) are coated with DNA and shot with a helium-driven gun at target tissues or cells (León and Fernández 2007). Mostly, this method has been applied in plants and, since 1990, also in microalgae (Mayfield and Kindle 1990). Microalgae transformants were achieved via particle bombardment in *C. reinhardtii* (Mayfield and Kindle 1990), *Chlorella sorokiniana* (Dawson *et al.* 1997) and *Chlorella kessleri* (El-Sheekh 1999).

***Agrobacterium*-mediated transformation** of microalgae result usually in stable transformants by applying the naturally occurring DNA-transfer of the *Agrobacterium*. Transfection of dicotyledons and monocotyledons are performed via a tumor inducing (T_i) plasmid. The T_i-DNA region of the T_i-plasmid are semi-randomly integrated into the genome of infected cells, which causes undifferentiated cell grows and tumor formation. Interestingly, *Agrobacterium* is also able to infect some algae without resulting in tumor development (Hallmann 2007). The first stable genome transformation of microalgae applying the *Agrobacterium*-mediated gene transfer was reported by Kumar *et al.* in 2004. In 2012, a protocol for *C. vulgaris* resulting in a transformation efficiency of up to 25 % was published (Cha *et al.* 2012).

As mentioned above, for some transformation methods the preparation of protoplasts prior to transformation is inevitable, as many microalgae species have a thick and tough outer cell wall, which decrease the transformation efficiency dramatically (Radakovits *et al.* 2010; Huang *et al.* 2006). Protoplast preparation is a very critical step, as the cells easily burst when exposed to shear forces, osmotic pressure, or other external influences during and after cell wall digestion. Also, rapid and easy regeneration of the cell wall after transformation is not guarantied (Huang *et al.* 2006). For those transformation methods, several cell-wall-deficient mutant strains were developed for *C. reinhardtii* (i. a. cw15; *Chlamydomonas Resource Center*). No cell-wall-deficient strain has been developed for other microalgae species (Kim *et al.* 2002).

To summarize this section regarding to *Chlorella* species, the most frequent applied transformation method is the electroporation (Maruyama *et al.* 1994; Huang *et al.* 2006; Chen *et al.* 2001; Chow and Tung 1999) and the PEG-method (Kim *et al.* 2002; Huang *et al.* 2006; Hawkins and Nakamura 1999), followed by the particle bombardment (Dawson *et al.* 1997; El-Sheekh 1999). No reports were found for *Chlorella* in which the SiC-method were applied. Just recently, a publication about an *Agrobacterium*-mediated transformation was published for *C. vulgaris*. The main problem of transformation of microalgae is the stable integration into the algal genome. Even when several working groups published about successful stable integration, not for all of them succeeded a complete and impeccable verification of integration event (Table 2.3). Suspicious is also, that none of them followed up with publications on engineered microalgae.

Table 2.3 | Transformation methods applied in *Chlorella* species and its verification for stable integration.

Transformation method	*Chlorella* species	Complete & impeccable verification of stable integration	Reference
electroporation	*Chlorella* sp. DT	no	Huang *et al.* 2006
	C. ellipsoidea	yes	Chen *et al.* 2001
	C. vulgaris	yes (some were tested positive, some not)	Chow and Tung 1999
	C. saccharophila	no	Maruyama *et al.* 1994
PEG-method	*Chlorella* sp. DT	no	Huang *et al.* 2006
	C. ellipsoidea	yes	Kim *et al.* 2002
	C. sorokiniana	no	Hawkins and Nakamura 1999
	C. vulgaris		
particle bombardment	*C. kessleri*	yes	El-Sheekh 1999
	C. sorokiniana	no	Dawson *et al.* 1997
Agrobacterium-mediated	*C. vulgaris*	no	Cha *et al.* 2012

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3. Material and Methods

3.1. Materials

In this section all materials used in this study are listed, including buffers and solutions, organisms and strains, media compositions, stock solutions (antibiotics, vitamins, etc.), kits, molecular weight standards, oligonucleotides (primers) and vectors. The experiments of this work were performed at two locations. When an experiment was performed at Northeastern University Boston or Leibniz University Hanover it will be labeled with the University abbreviation NEU or LUH, respectively.

3.1.1. Chemicals, Reagents and Enzymes

All applied chemicals correspond to the analytical degree of quality. If not specified, all chemicals, reagents and enzymes were purchased from the following companies:
AppliChem GmbH (Darmstadt, Germany), Biomol GmbH (Hamburg, Germay), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Chemicell GmbH (Berlin, Germany), Duchefa Biochemie B.V. (Haarlem, Netherlands), Fermentas (St. Leon-Rot, Germany), Finnzymes (Espoo, Finnland), Thermo Fisher Scientific Inc., (Waltham, USA), Implen (München, Germany), Invitrogen (Carlsbad, USA), Life Technologies (Invitrogen; Grand Island, USA), New England BioLabs (NEB, Ipswich, USA), PEQLAB Biotechnologie GmbH (Erlangen, Germany), Promega (Mannheim, Germany or Madison, USA), Qiagen N.V. (Hilden, Germany or Gaithersburg, USA), Roche Diagnostics AG (Mannheim, Germany), Sigma-Aldrich (Saint Louis, USA), Zymo Research (Irvine, CA, USA).

3.1.2. Buffers and Solutions

All in this study applied buffers and solutions were prepared with deionized water from the purification system Synergy (Millipore; Billerica, USA). For pH adjustments the pH-meter 827 pH lab (Metrohm; Filderstadt, Germany) were used. All buffers and solutions, not specifically mentioned in the protocols, are listed in the following Table 3.1.

Table 3.1 | Compositions of applied buffers and solutions.

Chapter	Working step	Buffer / Solution	Composition
general methods	agarose gel electrophoresis	50x TAE-buffer	2 M tris ultra pure; 50 mM EDTA, 5.71 % w/v acetic acid; ad 1 L H_2O
		RedSafe™ solution	10 % v/v stock solution in 0.5 % v/v TAE-buffer
		EtBr staining bath	0.5 - 1.0 µg/mL ethidium bromide (EtBr) in H_2O
	DNA resuspension or elution	Tris-H_2O	10 mM w/v tris base; adjust with HCl to pH 8.5

Chapter	Working step	Buffer / Solution	Composition
4	gDNA extraction	CTAB buffer	2 % w/v cyltrimethylammonium bromide (CTAB), 0.1 M tris base, 1.5 M NaCl, 10 mM EDTA, 0.2 % v/v ß-mercaptoethanol
		TE-buffer	0.1 M tris base, 10 mM EDTA
	yeast transformation	10x TE buffer	100 mM Tris, 10 mM EDTA
		10x LiAc buffer	1 M lithium acetate
		wash solution	1x LiAc buffer, 1x TE buffer
		PEG-solution	1x LiAc buffer, 1x TE buffer, 40 % w/v PEG-3370
	flow cytometry	PBS	137 mM NaCl, 12 mM phosphate, 2.7 mM KCl; pH 7.4
5	promoter evaluation	*GUS staining* solution	50 mM w/v Na_3PO_4, 0.2 % v/v triton-X100, 0.2 mM $K_3Fe(CN)_6$, 0.2 M $K_4Fe(CN)_6$, 1 mM X-Gluc
	N. benthamiana transformation	*seed-sterilization* buffer	0.5 % v/v sodium hypochlorite, 0.1 % v/v Tween20
		wash buffer	10 mM $MgCl_2$, 100 µM acetosyringone
		co-cultivation media	0.5 % MS (pH = 6), 1 % sucrose, 100 µM acetosyringone, 0.005 % Silvet L-77
	protoplast production	*wash* buffer	25 mM sodium phosphate buffer (pH 6)
		cell-wall-digestion buffer	25 mM sodium phosphate buffer, 0.6 M D-sorbitol, 0.6 M D-mannitol
	C. reinhardtii (Cr) & *C. vulgaris* (Cvu) transformation → PEG-method	Cr - *wash* buffer	0.3 M D-sorbitol, 0.3 M D-mannitol, 0.25 % w/v glucose in ES-media (Section 3.1.4.A)
		Cvu - *wash* buffer	0.3 M D-sorbitol, 0.3 M D-mannitol, 0.25 % w/v glucose in SH-media (Section 3.1.4.A)
		protoplast-solution	0.3 M D-sorbitol, 0.3 M D-mannitol, 50 mM $CaCl_2$
		PNC-solution	40 % v/v PEG-4000, 0.8 M NaCl, 50 mM $CaCl_2$
		Cr – *regeneration*-medis	0.3 M D-sorbitol, 0.3 M D-mannitol, 1 % yeast extract, 1 % glucose, in ES-media (Section 3.1.4.A); pH = 6.5
		Cvu – *regeneration*-media	0.3 M D-sorbitol, 0.3 M D-mannitol, 1 % yeast extract, 1 % glucose, in SH-media (Section 3.1.4.A); pH = 6.5
		Cr – expression-culture-media	0.3 M D-sorbitol, 0.3 M D-mannitol, 0.25 % w/v glucose, in ES-media (Section 3.1.4.A)
		Cvu – expression-culture-media	0.3 M D-sorbitol, 0.3 M D-mannitol, 0.25 % w/v glucose, in SH-media (Section 3.1.4.A)
	C. vulgaris: Agrobacterium-mediated transformation	*induction* media	BBM, containing 150 µM acetosyringone
		co-cultivation media plates	BBM, containing 150 µM acetosyringone, 0.125 mg/L biotin, 0.125 mg/L cyanocobalamin, 0.152 mg/L thiamin-HCl, 1.2 % w/v bacto agar

3.1.3. Organisms and Strains

In this study, four different organisms were used, including microalgae strains (*Chlorella vulgaris, Chlamydomonas reinhardtii*), one higher plant (*Nicotiana benthamiana*), yeast strains (*Saccharomyces cerevisiae* and derived mutants) and bacteria strains (*Escherichia coli, Agrobacterium tumefacience*). All organisms are listed in the table below.

Table 3.2 | Organisms and strains used in this study.

Class	Strain	Relevant genotype	Origin
microalgae	*C. vulgaris* UTEX-259	N/A	UTEX-The Culture Collection of Algae (Austin, USA)
	C. vulgaris UTEX-395	N/A	Dr. Stefanie Grade Leibniz University Hanover Institute of Biological Production Systems (Hanover, Germany)
	C. reinhardtii UTEX-2244	N/A	SAG Culture Collection (Goettingen, Germany)
bacteria	*E. coli* DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Invitrogen (Karlsruhe, Germany)
	E. coli NM522	F' *proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV thi-1 Δ(hsdS-mcrB)5*	Promega (Mannheim, Germany)
	A. tumefacience EHA105	contains the helper plasmid pSoup	N/A
yeast	*S. cerevisiae* SCY62	MATa ADE2 can 1-100 his3-11,15 leu2-3 trp1-1 ura3-1	Prof. Sten Stymme Swedish University of Agricultural Science (Sundsvägen, Sweden)
	S. cerevisiae H1262	*MATα are1-Δ :: HIS3 are2-Δ :: LEU2 lro1-Δ :: URA3 ADE2*	
	S. cerevisiae H1246	*MATα are1-Δ :: HIS3 are2-Δ :: LEU2 dga1-Δ :: KanMX4 lro1-Δ :: TRP1 ADE2*	
plant	*N. benthamiana*	N/A	N/A

N/A, not available.

All cloning steps were performed using two different *E. coli* strains of type K12 (DH5α and NM522). The promoter tests were performed with *N. benthamiana* (Chapter 5, Section 5.3.1.2.1.) and *C. reinhardtii* (Chapter 5, Section 5.3.1.2.2.) at LUH. For the functional characterization of CvuDGAT1 (Chapter 4, Section 4.3.4.), the three listed yeast strains were applied. The microalgal *C. vulgaris* UTEX-395 were used for transformation experiments performed at LUH. The main focus was set on *C. vulgaris* UTEX-259, cultured and maintained at NEU.

3.1.4. Media for Cultivation and Selection

The media for all cultures and selection media were sterilized at 121 °C and 1 bar pressure for 20 min. For the preparation of media plates the corresponding agar were added after pH adjustment. Sugars, vitamins and antibiotics (Section 3.1.5.) were added to the media after autoclaving.

(A) Media for Cultivation and Selection of Microalgae

For culturing and maintenance of *C. vulgaris* UTEX-259, Bold´s Basal Media (BBM) was used (Bold 1949). Its composition is given in Table 3.3. Stock solutions were prepared and stored at 4 °C. One day before sub-culturing the final culture media were prepared, adjusted to a pH of 6.6 and sterilized. Filter-sterilized vitamins and, if appropriate, antibiotics (Section 3.1.5.), were added prior to sub-cultivation. BBM-plates were produced with 0.8 % agar.

Table 3.3 | Composition of Bold´s Basal Media (BBM; Bold 1949).

			Stock solution (100 mL)	Final solution
	major solutions:			
1		$NaNO_3$	2.5 g	10 ml/L
2		$CaCl_2$ x $2H_2O$	0.25 g	10 ml/L
3		$MgSO_4$ x $7H_2O$	0.75 g	10 ml/L
4		K_2HPO_4	0.75 g	10 ml/L
5		KH_2PO_4	1.75 g	10 ml/L
6	**acidified iron solution:**			1 ml/L
		$FeSO_4$ x $7H_2O$	1.245 g	
		H_2SO_4	0.25 ml	
7	**trace metal solution:**			1 ml/L
		$ZnSO_4$ x $7H_2O$	2.205 g	
		$MnCl_2$ x $4H_2O$	0.36 g	
		MoO_3	0.178 g	
		$CuSO_4$ x $5H_2O$	0.393 g	
		$Co(NO_3)_2$ x $6H_2O$	0.123 g	
8	**baron stock solution:**		2.855 g	1 ml/L
9	**alkaline EDTA:**			1 ml/L
		EDTA Na x $2H_2O$	15.922 g	
		KOH	7.75 g	

C. reinhardtii were cultured and maintained in ES-media ("*e*rddekokt and salt" media[1]). *C. vulgaris* UTEX-395 were cultured in fertilized H_2O. After preparation, the media contained 0.6 g/L nitric nitrogen, 0.54 g/L ammonium nitrogen, 0.26 g/L carbamide nitrogen, 1 g/L phosphorus pentoxide (P_2O_5), 1.2 g/L potassium oxide (K_2O), 0.08 g/L magnesium oxide (MgO), 0.2 g/L sulfur, 0.002 g/L boron, 0.0004 g/L copper, 0.004 g/L iron, 0.002 g/L manganese, 0.0002 g/L molybdenum and 0.0004 g/L zinc. Both cultures were maintained by Dr. Stefanie Grade (LUH, Institute of Biological Production Systems).

(B) Media for Cultivation and Selection of Bacteria

For cultivation and selection of *E. coli* and *A. tumefaciens* several media were applied (Table 3.4). For preparation of solid media plates 1.5 % w/v agar were added.

Table 3.4 | Composition of applied media for bacteria cultures.

Media	Strain	Composition
2x TY	*E. coil*	1.6 % w/v tryptone, 1 % w/v yeast extract, 0.5 % w/v NaCl; pH 7.5
LB	*E. coil*	1 % w/v tryptone, 0.5 % w/v yeast extract, 1 % w/v NaCl; pH 7.5
YEP	*A. tumefacience*	1 % w/v tryptone, 1 % w/v yeast extract, 0.5 % w/v NaCl; pH 7.0
SOC	*E. coil* *A. tumefacience*	937.5 µL SOB*, 12.5 µL 2 M $MgCl_2$-solution,1 % w/v glucose
*SOB		2.0 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 2.5 mM KCl; pH 7.0

1 recipe: http://sagdb.uni-goettingen.de/culture_media/01%20Basal%20Medium.pdf; status: Sept. 20th 2014.

(C) Media for Cultivation and Selection of Yeast

For cultivation and selection of the three applied *S. cerevisiae* strains (SCY62, H1262, H1246; Chapter 4, Section 4.3.4.) different media were prepared (Table 3.5), including the general culture media YPD and two different compiled Dropout Base media (DOB-media; MP Biomedicals, Santa Ana, USA) supplemented with drop-out mix (DO-mix; amino acid mixture lacking histidine, leucine, tryptophan and uracil; Sigma Aldrich, St. Louis, USA) for selection and application in yeast experiments (Chapter 4, Section 4.3.4.). For media plates 1.7 - 2.0 % agar were added.

Table 3.5 | Composition of applied media for yeast cultures.

Media	Composition
YPD-media	2 % bactopeptone, 1 % yeast extract, 2 % glucose ; pH 6.5
SC[1]-media	2.7 % DOB powder, 0.8 g DO mix, histidine, leucine, tryptophan and uracil (Section 3.1.5.)
SC selection media	2.7 % DOB powder, 0.8 g DO mix, histidine, leucine, tryptophan (Section 3.1.5.)

[1] SC stands for *Saccharomyces cerevisiae*.

(D) Media for Growth and Selection of Nicotiana benthamiana

Sterile *N. benthamiana* seeds were spread on Murashige & Skoog media (MS-media; Duchefa Biochemie B.V., Haarlem, Netherlands) supplemented with 1 % sucrose and 0.8 % plant agar for seedling growth. The pH was adjusted to 5.8.

3.1.5. Stock Solutions (Antibiotics, Vitamins,...)

In this study, different types of substances where used, including antibiotics, vitamins, amino acids (aa), sugars and more. Their stocks solutions and commonly applied concentrations are listed in Table 3.6. For preparation the substances were solved in water, ethanol, DMSO, or chloroform. Stock solutions prepared with water were sterile-filtered with a Rotilabo 0.22 µm tip filter (Carl Roth; used at LUH) or 0.20 µm sterile filters (Corning, Inc., USA; used at NEU).

Table 3.6 | Applied stock solutions.

Type of substance	Substance	Solvent	Stock concentration	Commonly applied concentration
antibiotics	ampicillin	H_2O	50 mg / mL	50 µg / mL
	carbenicillin	H_2O	50 mg / mL	50 µg / mL
	kanamycin	H_2O	50 mg / mL	50 µg / mL
	hygromycin B	H_2O	50 mg / mL	50 µg / mL
	spectomycin	H_2O	50 mg / mL	several
	timentin	H_2O	100 mg / mL	100 µg / mL
	tetracycline	70 % v/v ethanol	5 mg / mL	50 µg / mL
	rifampicin	DMSO	5 mg / mL	5 µg / mL
vitamins	biotin	H_2O	125 mg / mL	125 µg / mL
	cyanocobalamin	H_2O	125 mg / mL	125 µg / mL
	thiamin-HCl	H_2O	125 mg / mL	125 µg / mL

Type of substance	Substance	Solvent	Stock concentration	Commonly applied concentration
amino acids	L-histidine	H_2O	100 mM	0.8 mM
	L-tryptophan	H_2O	40 mM	0.32 mM
	L-leucine	H_2O	100 mM	0.8 mM
	L-uracil	H_2O	20 mM	16 mM
sugars	D(+) glucose	H_2O	20 % w/v	several
	D(+) raffinose	H_2O	20 % w/v	several
	D(+) galactose	H_2O	20 % w/v	several
other	acetosyringone	DMSO	100 mM	100 µM
	IPTG	H_2O	100 mM	0.1 mM
	x-gal	DMSO	20 mg/mL	0.1 mg/mL
	x-gluc	DMSO	100 mM	2 mM
	RedSafe™	H_2O	1 % v/v	0.2 % v/v
	Bodipy® 493/503	chloroform	1 mg/mL	10 µg / mL

3.1.6. Kits

For the purification of DNA and PCR products (Chapter 4, Section 4.1.3.), but also for gel-purification, the Wizard® SV Gel and PCR Clean-Up System (Promega) or the Zymoclean™ Gel DNA Recovery Kit (Zymo Research) were used at LUH and NEU, respectively. Isolation of plasmids were performed with the peqGOLD Plasmid Miniprep kit (PEQLAB; used at LUH) or the Zyppy™ Plasmid Miniprep Kit (Zymo Research; used at NEU). For the preparation of the gDNA and cDNA library (Chapter 4) the GenomeWalker™ Universal Kit and the SMARTer™ RACE cDNA Amplification Kit (Clontech) were used, respectively. The cloning for identification of the *gene of interest* (*goi*; Chapter 4) were performed with the CloneJET PCR Cloning Kit (Fermentas; used at LUH) or the pGEM®-T Easy Vector System (Promega; used at NEU).

3.1.7. Molecular Weight Standards

DNA ladders were used for the estimation of DNA fragment sizes from plasmid isolations, PCR amplifications, or restriction digests from an agarose gel. At LUH, the GeneRuler™ 100bp DNA Ladder Plus or the GeneRuler™ 1kb DNA Ladder from Fermentas were applied. At NEU, the 1 kb and 100 bp DNA ladder from NEB were used.

3.1.8. Oligonucleotides

All oligonucleotides served as primers in PCRs (Chapter 4 and 5). They are listed in Table 3.7. The synthesis was performed either by Eurofins MWG Operon (for LUH) or IDT DNA (for NEU). The primer numbers (#) refer to our personal consecutive numbering for oligonucleotides.

Table 3.7 | In this study applied oligonucleotides.

#	Name	Sequence	Application	Chapter
3	CvaCR**1**dgat1_f	TCA AGC TGG TGT CGT ATG	Primers for the nested PCR to identify *Cvudgat1* sequence parts.	4
33	CvaCR**2**dgat1_f	CTA ATC TAC CAG CTC ACC TAC CCG C		
34	CvaCR**3**dgat1_r	CAG TCC TTG TAG AAC TCG CGG TC		
21	CvaCR**4**dgat1_r	GGA AGT AGA CGT GTC GCA GCA TCC ACT TG		
56	Cvudgat1**m**_f	TGC GCC ATC ATC CTG CAC ACC TG	*Cvudgat1* 5´- and 3´-end amplification.	4
41	Cvudgat1**m**_r	GGT GTA GAA CAT GGC CAG CCA CCA GTA GAG		
54	Cvudgat1_f	ATG GCG CCC CGC GCA AGC CTG	Amplification of the entire *Cvudgat1* gene.	4
55	Cvudgat1_r	TCA GAT AAT GGG CGT GCC CTG GAA G		
15	pGII_MAS/GUS_f	GGC GAG CTC ACG ACT CAC TAT AGG	Amplification of MAS_GUS for insertion into the pUC18 cloning vector, the forward and reverse primer contain the SacI and AflII RE sites, respectively.	5
16	pGII_MAS/GUS_r	GGC CTT AAG AAC CGA ATC GAA GC		
13	RBCS2pCB740_r	GCG TCT AGA GTG CTA GCT CTC TTG	Amplification of the three promoters RBCS2, Ubi1 and Hsp70A/RBCS2 from their provided vectors to exchange the MAS promoter within the pUC18_MAS_*gus* cloning vector → Ubi1 primer_28/29 → Hsp70A/RBCS2 primer_30/31 → RBCS2 primer_14/13 Primer_13 and primer_14 contain a XbaI and ClaI (Bsu15I) RE site, respectively.	5
14	RBCS2pCB740_f	GGC ATC GAT ACA TAA CCA CTC AGC		
28	OEpUC18Ubi1_f	CAC TAT AGG GCG AAT TGG GTA CCG GGC CCC CCC TCG AGG TCG ACG GTA TCG CTT GAA GCG GAG GTG CCG		
29	OEpUC18Ubi1_r	CGG TGT TTT GGT TTT TTC TTG TGG CCG TCT TTG TTT ATA TTG AGA TCT GCA GTG CAG CGT GAC C		
30	OEpUC18HR_f	CAC TAT AGG GCG AAT TGG GTA CCG GGC CCC CCC TCG AGG TCG ACG GTA TCG GGA TCC ACT ATA GGG CGA ATT G		
31	OEpUC18HR_r	CGG TGT TTT GGT TTT TTC TTG TGG CCG TCT TTG TTT ATA TTG AGA TCT CTC TTG TAA AAA AGT AAA GAA CAT AGG		
45	SacIGUS_f	CAT GAG CTC TTG TTT GCC TCC CTG	Amplification of the *promoter_gus* constructs out of pUC18 for cloning into the final vector pMDC162_w/oGWsys → MAS_*gus* primer_47/45 → Ubi1_*gus* primer_48/45 → Hsp70A/RBCS2_*gus* primer_49/46 → RBCS2_*gus* primer_50/45 Primer_45 contains a SalI RE site, primer_46/47/48/50 a KpnI RE site and primer_49 a PstI RE site.	5
46	KpnIGUS_f	CAT GGT ACC TTG TTT GCC TCC CTG		
47	KpnIMAS_r	CCC GGT ACC ATG ATT TGG TGT ATC G		
48	KpnIUbi1_r	CAA GGT ACC CTT GAA GCG GAG GTG		
49	PstIHR_r	GGT CTG CAG GGA TCC ACT ATA GG		
50	KpnIRBCS2_r	CCC GGT ACC ATA CAT AAC CAC TCA G		
59	DGATKpn2I_f	GTA TCC GGA ATG GCG CCC CGC GCA AGC CTG	Amplification of the *Cvudgat1* sequence with for the insertion into the vector pCIPG. Primer_59 contains a Kpn2I and primer_60 a HindIII RE site.	4
60	DGATHindIII_r	GGG AAG CTT GAT AAT GGG CGT GCC CTG GAA G		

Over- and underlined nucleotides within the primer sequences label recognition sites for restriction enzymes (RE sites).

3.1.9. Vectors

In this work several vectors were used (Table 3.8), including plasmids providing the promoters, containing a strong expression cassette, but also cloning vectors. The final pMDC162 vectors, including the expression cassettes, shall be used for recombinant gene expression in *C. vulgaris* (Chapter 5).

Table 3.8 | Overview of the vectors used and compiled in this study.

Vector	Size (bp)	Utilization	Source	Chapter
pYES2	5,856	functional characterization of *Cvudgat1*	Christoph Benning Michigan State University Department of Biochemistry and Molecular Biology (East Lansing, USA)	4
pYES2_*Cvudgat1*	7,163		this work	4
pCAMBIA1305.1_hygB_MAS_*gus-luc*	8,807	provides the MAS_*gus* construct	Heiko Kiesecker Leibniz Institute, DSMZ[1] (Braunschweig, Germany)	5
N/A[2]	N/A	provides the Ubi1 promoter	Sean Flanigan, Interim Director of Technology Transfer & Business Enterprise of the University of Ottawa (Canada)	5
pCB740	16,647	provides the Hsp70A/RBCS2 and the RBCS2 promoter	*Chlamydomonas* Resource Center University of Minnesota (USA)	5
pUC18	2,686	cloning vector	Thermo Fisher Scientific (Schwerte, Germany)	5
pUC18_MAS_*gus*	5,036	*promoter_gus* constructions	this work	5
pUC18_Ubi1_*gus*	5,636		this work	5
pUC18_Hsp70A/RBCS2_*gus*	5,178		this work	5
pUC18_RBCS2_*gus*	4,775		this work	5
pMCD162	12,886	binary vector containing a Gateway system (GWsys)	*Arabidopsis* biological resource center (ABRC) Ohio State University (Columbus, USA)	5
pMCD162_w/oGWsys	9,244	binary vector without GWsys	this work	5
pMCD162_w/oGWsys_MAS_*gus*	11,574	constructed vectors for promoter evaluation	this work	5
pMCD162_w/oGWsys_Ubi1_*gus*	12,174		this work	5
pMCD162_w/oGWsys_Hsp70A/RBCS2_*gus*	11,695		this work	5
pMCD162_w/oGWsys_RBCS2_*gus*	11,313		this work	5
pCleanIPG_2x35S-smGFP_ER	7,237	provides strong 5´and 3´ UTR with additional 3´ added tags for identification and purification of the synthesized protein	Thomas Reinard Leibniz University Hanover Institute for Plant Genetics (Hanover, Germany)	5
pMCD162_w/oGWsys_MAS_*Cvudgat1*	11,574	**final vectors** for overexpression of *Cvudgat1* in *C. vulgaris*. → vector map is shown in Figure 3.1	this work	5
pMCD162_w/oGWsys_Ubi1_*Cvudgat1*	12,174		this work	5
pMCD162_w/oGWsys_Hsp70A/RBCS2_*Cvudgat1*	11,695		this work	5
pMCD162_w/oGWsys_RBCS2_*Cvudgat1*	11,313		this work	5

[1] DSMZ – *German Collection of Microorganisms and Cell Cultures*
[2] N/A not assigned

Final pMDC162 vectors:

The original pMDC162 vector was derived form the pCAMBIA vectors (Curtis and Grossniklaus 2003). In the pMDC162 backbone are located the kanamycin resistance gene (neomycin phosphotransferase II; *nptII*) for bacterial selection, the *E. coli* replicon pBR322 and the *A. tumefaciens* pVS1 origin. The T_i-region of the pMDC162 contains the hygromycin B resistance gene (hygromycin phosphotransferase II; *hptII*) driven by a two-fold CaMV 35S promoter in reverse direction, a nopaline synthase (nos-) terminator, several tags (flag-, strepII-, and 6x his-tag), the KDEL sequence, the *gusA* gene and the Gateway system. As the Gateway system and the *gusA* gene were not needed in this study, it was removed with SacI (cuts twice) digestion and re-ligation. The vector is now called pMDC162_w/oGWsys. The final high-powered expression cassettes were inserted in to this new vector, containing the four promoters (MAS, Ubi1, Hsp70A/RBCS2 and RBCS2) in driving the *goi*, *Cvudgat1* (Chapter 5; Figure 3.1).

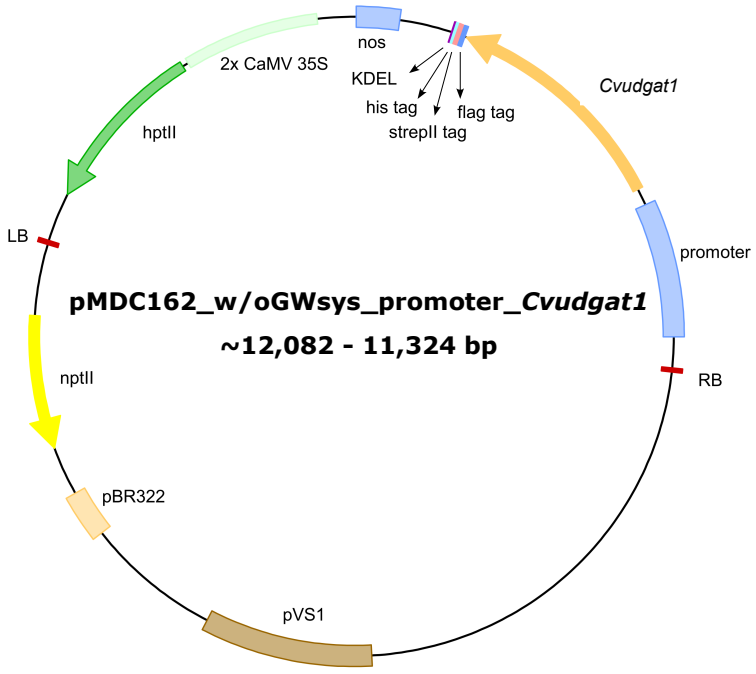

Figure 3.1 | Vector map of the final vector/s of this study – pMDC162_w/oGWsys_*promoter_Cvudgat1*. At the position of the *promoter* in this map occurs either the MAS, Ubi1, Hsp70A/RBCS2, or the RBCS2 promoter. The size range refers to the promoter size. The vector sizes are 11,482 bp, 12,082 bp, 11,624 bp and 11,324 bp for containing the MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 promoter, respectively. ***Cvudgat1***: diacylglycerol acyltransferase type-1 gene from *C. vulgaris*; **nos**: nos terminator; ***nptII***: kanamycin resistance gene encoding neomycin phosphotransferase II; ***hptII***: hygromycin B resistance gene encoding hygromycin phospho-transferase II; **pBR322**: replicon for *E. coli*; **pVS1**: replicon for *A. tumefaciens*; **RB**: right border; **LB**: left border.

3.2. Methods

3.2.1. Cultivation and Maintenance

In this section, all methods concerning culture maintenance and conditions were described, including cultivation procedures for microalgae, bacteria and yeast, but also the determination of the optical density of cultures and the preparation of culture for long-time-storage (glycerol stocks) are listed.

3.2.1.1. Cultivation of C. vulgaris UTEX-259

C. vulgaris cells were cultured in Bold´s Basal Media (BBM; Bold 1949), modified by (Starr and Zeikus 1993). Cultures were maintained at 26 ± 3 °C, with 16 hours light per day and 110 rpm mixing rate in an incubator shaker (Forma Scientific, Marietta, OH, USA) modified to supply light from the shaker platform. The light (300 - 400 µE/m^2/s) was provided by three 20 W cool white fluorescent bulbs (Microfluorescent T4 lights with color temperature of 4100 K, Pegasus Associates, PA, USA). Maintenance cultures (400 mL of culture in 1 L flasks capped with foam closures; Bellco Glass, Vineland, NJ, USA) were diluted in half with fresh BBM every seven days to maintain the cultures in exponential growth with an initial cell density of 2 - 4x10^7 cells/mL.

3.2.1.2. Cultivation of C. vulgaris UTEX-395 and C. reinhardtii UTEX-2244

Dr. Stefanie Grade of the Institute of Biological Production Systems (LUH) took over the cultivation and maintenance of *C. vulgaris* and *C. reinhardtii*. The media compositions are described in Section 3.1.4.A. Both cultures were cultivated at 24 ± 2 °C by agitating at 180 rpm at a photon flux density of 50 µE/m^2/s provided by fluorescent bulbs.

3.2.1.3. Cultivation of Bacterial Cultures

The cultivation of *E. coli* and *A. tumefaciens* were performed in liquid but also on media plates (Section 3.1.4.B). Liquid cultures were maintained in 50 mL reaction tubes, containing 10 mL media supplemented with antibiotics (Section 3.1.5.). The media were usually inoculated with a picked colony or 100 µL of a glycerol stock (Section 3.2.1.6.). For cell growth, *E. coli* and *A. tumefaciens* cultures were incubated on a shaker at 180 - 220 rpm and 37 °C and 28 °C, respectively, for 6 to 18 hours. The cultivation on solid media were performed either by picking a colony and streaking it on plates, or by taking 50 - 100 µL of cell suspension to spread it with a spatula or glass-beads (Ø = 5 mm) on plates. For *E. coli* growth, the plates were stored at 37 °C over night. The incubation of *A. tumefaciens* were performed at 28 °C for two days.

3.2.1.4. Cultivation of Yeast Cultures

For the cultivation of the untransformed *S. cerevisiae* strains SCY62, H1262 and H1246 YPD-media were used (Section 3.1.4.C). These strains were sub-cultured on YPD-plates every two to three weeks. Therefore, a picked colony were re-streaked on new YPD-plates, incubated at 30 °C for two days and stored in the fridge. For liquid cultures, a colony of the sub-culture plate were picked for

inoculation of 10 mL YPD-media in a 50 mL reaction tube. The approaches were incubated on a shaker at 220 rpm and 30 °C for up to two days.
Transformed yeast cells were cultured in SC-selection media lacking uracil (Section 3.1.4.C) under the same culture conditions. For experimental approaches the untransformed strains served as controls and were cultivated in SC-media (Section 3.1.4.C).

3.2.1.5. Determination of the Optical Density of Single Cell Cultures

The optical density (OD) of bacterial, algal and yeast suspensions were determined photometric using the NanoPhotometer™ (Implen) by detection at the wavelength of 600 nm. The corresponding culture media served as reference.

3.2.1.6. Preparation of Glycerol Stocks

To store bacterial, algal and yeast cultures for a long time, glycerol stocks were prepared. Therefore, a fresh grown culture were transferred in a 2 mL tube and mixed with 86 % v/v glycerol to a final concentration of 25 - 50 % glycerol. The samples were shock-frozen in liquid nitrogen and stored at -80 °C.

3.2.2. Nucleic Acid Extraction Protocols

The methods described deal with the extraction of nucleic acids (plasmid DNA, genomic DNA, mRNA), but also with library preparation (*C. vulgaris* cDNA and gDNA libraries). Additionally, methods for quantification, purification and sequencing of nucleic acids are described.

3.2.2.1. Plasmid Isolation from E. coli

Plasmid isolation from *E. coli* were performed based on the principle of the alkaline lysis according to Sambrook and Russell (2001). Then recombinant plasmids were supposed to be sequenced (Section 3.2.2.8.), the isolation was performed with a plasmid isolation kit (Section 3.1.6.).

3.2.2.2. gDNA Isolation from C. vulgaris

The gDNA isolation were performed for *C. vulgaris* according to the protocol provided by Dawson *et al.* (1997) and Chen *et al.* (2001). Their protocols are based on the CTAB-method for the gDNA extraction form higher plants. For cellular disruption, 10 mL of a 7 days old *C. vulgaris* culture (1 - 2 x 10^8 cells/mL) was harvested by centrifugation (3,000 xg, 3 min) and shock-frozen in liquid nitrogen. While thawing the sample, 500 µL of CTAB-buffer (Section 3.1.2., Table 3.1) was added. The cells were disrupted using a bead beater with each vial ¼ full of glass-beads (230 VAC, 50 Hz, Mini-Beadbeater-1, Biospec Products; NEU). Each cycle consisted of shaking at 5,000 rpm for 30 s followed by 1 min on ice and then repeated three times. All following steps were performed as described by Dawson *et al.* (1997) and Chen *et al.* (2001). Recipes for the solution are listed in Section 3.1.2. Table 3.1.

3.2.2.3. mRNA Isolation from C. vulgaris

For mRNA isolation, a 7 day old *C. vulgaris* culture were used grown up to a density of 1 - 2 x 10^8 cells/mL. The cell suspensions were transferred into a plastic bag, which was placed in a 50 mL reaction tube (Figure 3.2). Cells were harvested by centrifugation at 2,000 xg for 3 min. After discarding the supernatant, the step was repeated until 50 mL culture were harvested. The plastic bag within the cell pellets was then shock-frozen in liquid nitrogen. After the bag was broken open, the frozen microalgae cell pellets were grinded with mortar and pestle. The still frozen algal powder were transferred into a 1.5 mL tube containing 1 mL RNAzol™ RT (Sigma-Aldrich®). After a short mixing step, 400 µL H_2O per 1 mL RNAzol® RT was added to the sample, incubated for 15 min at room temperature (RT) and centrifuged at 12,500 xg for 15 min. Then, 1 mL of the supernatant were transferred into a new 1.5 mL reaction tube, mixed with 400 µL of 75 % v/v ethanol and stored for 10 min on ice, followed by a further centrifugation step at 12,000 xg for 8 min. The pellets were washed twice with 400 µL of 75 % v/v ethanol (8,000 xg, 3 min), before removing the ethanol completely. The pellet were resuspended in 20 µL H_2O. The quality and quantity of the mRNA were determined by photometric measurements (Section 3.2.2.6.).

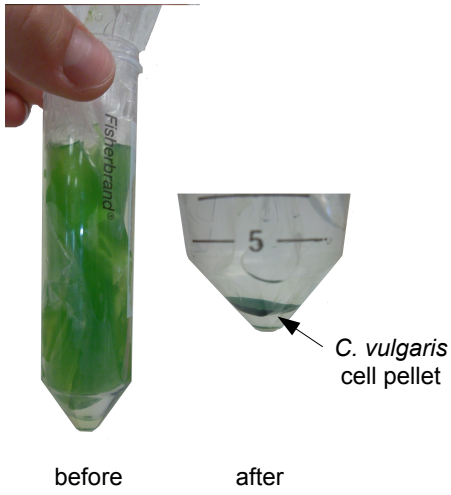

Figure 3.2 | Harvest of *C. vulgaris* for mRNA isolation. Pictures were made right before and after centrifugation.

3.2.2.4. Construction of a C. vulgaris gDNA Library

The gDNA of *C. vulgaris* was extracted using the CTAB-method (Section 3.2.2.2.). gDNA libraries from *C. vulgaris* were prepared using the GenomeWalker™ Universal Kit (Clontech, USA).

3.2.2.5. Construction of a C. vulgaris cDNA Library

The mRNA was extracted using RNAzol® RT (Section 3.2.2.3.). The SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) was used to synthesize the cDNA and prepare the 5´- and 3´- end cDNA libraries.

3.2.2.6. Quantification and Qualification of Nucleic Acids

The quality and quantity of DNA- and RNA-solutions were determined by photometric measurements performed with a NanoPhotometer™ (Implen; used at LUH) or with a NanoDrop® ND-1000 (Thermo Scientific; used at NEU). The re-suspension or elution buffer served as reference.

3.2.2.7. Purification of DNA-Fragments

The purification of DNA fragments was necessary for the removal of enzymes, the change of buffer conditions, for gel purification, or prior to sequencing. Therefore, the *Wizard® SV Gel and PCR Clean-*

Up System (Promega; used at LUH) or the *Zymoclean™ Gel DNA Recovery Kit* (Zymo Research, used at NEU) were applied (Section 3.1.6.).

3.2.2.8. DNA-Sequencing

For sequencing, purified plasmid DNA was sent to GATC Biotech (Konstanz, Germany) or GENEWIZ (Boston, USA).

3.2.3. Polymerase Chain Reaction

The polymerase chain reaction (PCR) were used to amplify DNA-fragments for cloning with the *Phusion*™- or Phusion® High-Fidelity DNA-Polymerase (Finnzymes, NEB). The GoTaq DNA-Polymerase (Fermentas, NEB) were used to identify positive transformants (colony-PCR) or to prove constructs. The reactions were performed with a thermocycler from SensoQuest (used at LUH) or with the Mastercycler® from Eppendorf (used at NEU).

3.2.3.1. Standard-PCR

The standard-PCR describes a common amplification procedure to obtain large quantities of a desired DNA-fragment. Therefore, typically the Phusion DNA-Polymerase was used. A general layout for a 20 µL approach is shown in Table 3.9.

Table 3.9 | General layout and conditions for a standard PCR with the Phusion DNA-Polymerase.

Approach		Reaction conditions			
Compounds	**Volume**	**Step**	**Temperature**	**Time**	
5x Phusion HF buffer	10.0 µL	initial denaturation	98 °C	30 s	
10 mM dNTPs	1.0 µL	denaturation	98 °C	10 s	35x
10 µM primer_forward	x µL	annealing	x °C	30 s	
10 µM primer_reverse	x µL	elongation	72 °C	30 s / kb	
DNA template	x µL	final elongation	72 °C	10 min	
Phusion DNA-Polymerase	0.5 µL	storage	4 °C	∞	
H_2O	ad 20.0 µL				

When the annealing temperature of the primers was ≥ 72 °C a two-step-PCR was performed. Therefore, the annealing and elongation steps were combined, resulting in an extended 72 °C step.

3.2.3.2. Colonie-PCR

The colony-PCR was used as fast verification method to identify putative transformants after cloning. Colonies from the transformation plate were picked, transferred to 200 µL reaction tubes containing 20 µL H_2O and heated up to 95 °C for 5 min. After a short centrifugation step, 7 µL was transferred into a 13 µL PCR approach (Table 3.10). For this PCR the *GoTaq* DNA-Polymerase was applied.

Table 3.10 | General layout and conditions for a colonie-PCR.

Approach		Reaction conditions			
Compounds	**Volume**	**Step**	**Temperature**	**Time**	
5x GoTaq Puffer	4.0 µL	denaturation	94 °C	30 s	30x
25 mM $MgCl_2$	2.0 µL	annealing	x °C	30 s	
10 mM dNTPs	0.5 µL	elongation	72 °C	1 min 30 s	
10 µM primer_forward	0.5 µL	final elongation	72 °C	10 min	
10 µM primer_reverse	0.5 µL	storage	4 °C	∞	
GoTaq DNA-Polymerase	0.2 µL				
H_2O	ad 13.0 µL				

3.2.3.3. Nested-PCR

The nested-PCR were applied for identification of sequence parts of a *goi*, such as diacylglycerol acyltransferase type-1 (*dgat1*; Chapter 4). Therefore, two PCRs were performed, with one primer set for each reaction. In the primary PCR, the first primer set was used (primer_A+B) with the *C. vulgaris* cDNA or gDNA library as template (Section 3.2.2.4. and 3.2.2.5.). The secondary PCR was prepared with the primary PCR as template and the second primer set (primer_C+D). At this stage, the primer_C+D bind within the sequence amplified by the first primer set (primer_A+B). This was made to improve the specificity of the product. For both PCRs, the protocol of the standard-PCR were applied (Section 3.2.3.1.).

3.2.3.4. Agarose Gel Electrophoresis

Electrophoresis was used to separate DNA of different fragment sizes in an agarose gel, for example, after a PCR, following restriction digestion (Section 3.2.4.1.), or to verify plasmids. Therefore, gels containing 0.5 to 1.2 % w/v agarose in 0.5x (LUH) or 1 x TAE-buffer (NEU; Section 3.1.2., Table 3.1) were used appropriate to the expected fragment sizes. To the DNA samples were added 6x leading dye (Fermentas, NEB) before loading the gel, when not provided in the reaction buffer. The gel ran in the appropriate TAE-buffer system with 100 V or 130 V for 25 to 60 min. At LUH, we used RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea) to visualize DNA. The stain was added directly to the gel right before pouring. At NEU, we used ethidium bromide (EtBr; Section 3.1.2., Table 3.1). After electrophoresis the gel was incubated for 5 to 15 min in the EtBr-bath before documentation.

3.2.4. Methods for Construction of Recombinant Plasmids

Here, all applied methods for recombinant plasmid construction are listed, including those applied for classic cloning, but also the overlap-extension PCR cloning strategy. The transformation methods are excluded in this section, but described in the following Section 3.1.5..

3.2.4.1. Restriction Enzyme Digestion of DNA

The enzymatic digestion of DNA-fragments or plasmids were performed with FastDigest restriction endonucleases (Fermentas, NEB). Commonly, a 20 µL approach were prepared, including the provided FastDigest-buffer, the DNA and 1 µL of the corresponding restriction enzyme/s. The reaction were performed in the same thermocycler used for PCR (Section 3.2.3.) at 37 °C for 20 min up to two hours, except for Kpn2I, which required an incubation temperature of 55 °C to cut best. When the digestion was used for further approaches, the enzyme/s were inactivated in 10 min at 80 °C.

3.2.4.2. A-tailing of DNA-Fragments

The pJET1.2 or pGEM®-T cloning vectors (Section 3.1.6.) were provided linearized with a 5´- thymine (T-) overhang. Consequently, the DNA-fragment requires a 3´ - adenine (A-) overhang for insertion. Some DNA-Polymerases, such as the Crimson-Taq-Polymerase (NEB, USA), automatically add a 3 ´ - A-overhang on the end of a synthesized strand. Hence, after amplification of the insert with a standard-PCR, an A-tailing were performed with 9.5 µL of purified PCR product in 3 µL of the provided 5x buffer, 1 µL Crimson-Taq-Polymerase and 1µL 1 mM dATPs. The approach were incubated at 68 °C for 10 min and directly used for ligation (Section 3.2.4.4.).

3.2.4.3. Dephosphorylation of DNA-Fragments

The dephosphorylation of vectors prevents from its re-ligation in cloning experiments. Therefore, the Shrimp Alkaline Phosphatase (SAP; Fermentas, NEB) were used. It were added to the vector approach with its provided buffer and were incubated for 30 min at 37 °C, followed by an inactivation step at 65 °C for 15 min.

3.2.4.4. Ligation

The ligation of DNA-fragments were performed with T4 DNA Ligase (Fermentas, NEB) in its provided buffer system and with additional 1 µL 100 mM ATP suspension (Fermentas). The vector to insert ration as chosen to 1:5 in a final volume of 20 µL. The ligation reaction occurred at 22 °C for two hours or at 16 °C over night, followed by an inactivation step at 65 °C for 10 min. The ligation was directly used for transformation (Section 3.2.5.1.).

3.2.4.5. Overlap-Extension-PCR Cloning

The overlap-extension (OE-) PCR is a cloning method without the need of restriction enzymes applied in classic cloning approaches (Bryksin and Matsumura 2010). This method finds application when no suitable RE sites are available. This PCR or cloning strategy were performed in three steps. In the first step, a megaprimer were amplified containing the insert (Table 3.11). Followed by the OE-PCR, in which the megaprimers were used to introduce the insert into the vector by amplification of its backbone. The elongation time in this PCR is highly extended corresponding to 90 s per kb vector (Table 3.12). In this step the exact insert to vector ratio is most crucial. It can be calculated with a simple formula by consideration of the vector and insert length as shown below. In the last step, prior to *E. coli* transformation, a DpnI digest were performed to eliminate the original vector (inserted vector without insert). An overview and further explanations of this cloning method are given in the Appendix (Supplementary Material 3.I).

Table 3.11 | Layout and conditions for the megaprimer amplification.

Approach		Reaction conditions			
Compounds	**Volume**	**Step**	**Temperature**	**Time**	
5x Phusion HF buffer	4.0 µL	initial denaturation	98 °C	2 min	
10 mM dNTPs	0.4 µL	denaturation	98 °C	30 s	30x
10 µM OE-primer_forward	0.5 µL	annealing	x °C	30 s	
10 µM OE-primer_reverse	0.5 µL	elongation	72 °C	90 s/kb	
3-30 ng template	x µL	final elongation	72 °C	10 min	
Phusion DNA-Polymerase	0.2 µL	storage	4 °C	∞	
H_2O	ad 20.0 µL				

→ formula for the calculation of vector to insert ratio:

$$\frac{ng(megaprimer) \;/\; bp(megaprimer)}{ng(vector) \;/\; bp(vector)} = 250$$

Table 3.12 | Layout and conditions for the OE-PCR.

Approach		Reaction conditions			
Compounds	**Volume**	**Step**	**Temperature**	**Time**	
5x Phusion HF buffer	..0 µL	initial denaturation	98 °C	2 min	
10 mM dNTPs	1.0 µL	denaturation	98 °C	30 s	17x
megaprimer (contains insert)	x µL	annealing	x °C	30 s	
template (target vector)	x µL	elongation	72 °C	90 s/kb	
Phusion DNA-Polymerase	0.2 µL	final elongation	72 °C	10 min	
H_2O	ad 10.0 µL	storage	4 °C	∞	

For the final digestion of the original vector, 10 units DpnI were added to each OE-PCR approach and incubated for one hour at 37 °C before the enzyme were inactivated in 20 min at 80 °C. The approaches were direcly used for *E. coli* transformation (Section 3.2.5.1.).

3.2.5. Transformation Protocols

This section comprises protocols used for transformation of bacteria, yeast, *N. benthamiana* and microalgae.

3.2.5.1. Transformation of E. coli

First, chemically competent *E. coli* cells were prepared. Therefore, a fresh grown culture with a OD_{600} between 0.3 and 0.4 were harvested by centrifugation (4.500 xg, 4 °C, 10 min). After discarding the supernatant, the cell pellet were resuspended in half of the volume with ice-cold 100 mM $CaCl_2$ and stored for 20 min on ice. Thereupon, a second centrifugation step were performed (same settings), followed by resuspension in one tenth of the initiated volume with 100 mM $CaCl_2$. After 60 min of incubation on ice, the cells were directly used for transformation or treated with 86 % v/v glycerol (15 % final concentration), shock-frozen in liquid nitrogen and stored at -80 °C.

For the transformation of 50 µL chemically competent *E. coli* cells either 0.5 - 1 µL plasmid DNA suspension or 2 - 5 µL ligation approach (Section 3.2.4.4.) were added to the cell suspension and incubated for 20 min on ice. The approach were heat shocked at 42 °C for 45 s and transferred back on ice for 2 min before adding 200 - 400 µL pre-warmed SOC-media (Section 3.1.4.B, Table 3.4). The cells were incubated on a shaker at 180 rpm (LUH) or 220 rpm (NEU) and 37 °C for 30 to 60 min, streaked on antibiotic-supplemented 2x TY media plates (Section 3.1.4.B, Table 3.4) and incubated over night at 37 °C.

3.2.5.2. Transformation of A. tumefaciens

Prior to transformation, electro-competent *A. tumefaciens* cells were prepared. Therefore, a fresh grown culture (50 mL) with a OD_{600} of 0.4 to 0.5 were harvested by centrifugation (4.500 xg, 4 °C, 5 min). The pellet was washed twice with 25 mL ice-cold 10 % v/v glycerol (complete resuspension of the pellet each time) and were then resuspended carefully in 5 mL 10 % v/v glycerol. The cells were directly used for transformation or treated with 86 % glycerol (15 % final concentration), shock-frozen in liquid nitrogen and stored at -80 °C.

The transformation of 50 µL competent *A. tumefaciens* cells were performed with 1 µL plasmid DNA. Therefore, the suspension were transferred into a pre-cooled cuvette with 1 mm electrode distance and stored on ice. The electroporation of the cells were performed with a MicroPulser™ Electroporator (BioRad; LUH) with a capacitance at 25 µF, a resistance of 200 Ω and an electric pulse of 2500 V/cm. After the electric shock, 450 µL SOC-media (Section 3.1.4.B, Table 3.4) were added and the approaches transferred into a 2 mL reaction tube, followed by an incubation on ice for 30 min and then for three hours on a shaker at 220 rpm at 28 °C. The cell suspensions were plated on YEP-media plates supplemented with the corresponding antibiotic (Section 3.1.4.B, Table 3.4) and stored over two days at 28 °C.

3.2.5.3. Transformation of Yeast

The yeast were transformed by the lithium acetate (LiAc-) method (Gietz and Woods 2002). The recipes for the required solutions are listed in Section 3.1.2., Table 3.1.

3.2.5.4. FAST-Method for N. benthamiana Seedling-Transformation

The transformation of *N. benthamiana* seedlings were performed by the Fast *Agrobacterium*-mediated Seedling Transformation protocol (FAST-method) according to (Li *et al.* 2009). First, 50 mg of *N. benthamiana* seeds were sterilized in *seed-sterilization* buffer (Section 3.1.2., Table 3.1.) for 10 min at RT. After discarding the liquid phase, the seeds were incubated for 1 min at 70 % v/v ethanol while continuous shaking and washed five times with sterile H_2O for 30 sec each. For germination, the seeds were transferred to MS-media plates (Section 3.1.4.B) and exposed to 16 hours light a day at 22 °C in a climatic chamber. Eight days old seedlings were used for transformation according to Li *et al.* (2009). For transformation, the *N. benthamiana* seedlings were cultivated in *co-cultivation* media (Section 3.1.2., Table 3.4.) with *A. tumefaciens,* containing the wanted constructs, including the *goi*, for 50 hours in the dark. For expression of the *goi*, the approaches were stored with 16 hours light a day at 22 °C for 24 hours before evaluation (Chapter 5, Section 5.3.1.2.1.).

3.2.5.5. Microalgal Protoplast Production

The microalgal transformation via PEG-method (Section 3.2.5.6.) requires a prior formation of protoplasts. Therefore, 50 mL of a fresh grown microalgal culture (2 x 10^7 cells/mL) were harvested by centrifugation (1,500 xg, 5 min). The cell pellet was washed once with *wash* buffer (Section 3.1.2., Table 3.4.) and were then resuspended in *cell-wall-digestion* buffer (Section 3.1.2., Table 3.4.). The enzymes cellulase (4 % w/v), macerase (2 % w/v) and pectinase (50 units) were added to the *cell-wall-digestion* buffer right before its application. The cell-digestion approach was incubated for 16 hours at 25 °C in the dark by gentle shaking (50 rpm). The formed protoplasts were used directly for transformation (Section 3.2.5.6.).

3.2.5.6. PEG-method for Microalgal Transformation

The PEG-method were performed for *C. reinhardtii* and *C. vulgaris* according to Kim *et al.* (2002). The protoplasts (Section 3.2.5.5.) were harvested by centrifugation (400 xg, 5 min) and washed once with the corresponding *wash* buffer (Section 3.1.2., Table 3.4.). The cells were gently resuspended in 1 mL *protoplast*-solution (Section 3.1.2., Table 3.4.) and distributed in 140 µL aliquots into new 1.5 mL reaction tubes. Then, 5 µg plasmid DNA and 25 µg calf-thymus DNA were added. The approaches were incubated for 15 min at RT before adding 200 µL of pre-cooled *PNC*-solution (Section 3.1.2., Table 3.4.). The suspensions were incubated for 30 min on a rocker. Finally, 600 µL *regeneration*-media (Section 3.1.2., Table 3.4.) were added and incubated for 23 hours at 25 °C in the dark.

For expression, *C. vulgaris* cultures were transferred into a 50 mL reaction tube with 15 mL of Cvu-*expression-culture*-media (Section 3.1.2., Table 3.4.). For *C. reinhardtii* expression cultures, the regenerated cells were transferred with 1 mL Cr-*expression-culture*-media in a 15 mL tube. The approaches were incubated on a shaker at 120 rpm and 25 °C for 30 hours.

3.2.5.7. Electroporation of C. vulgaris

For electroporation of *C. vulgaris,* 20 mL of a culture (2 x 10^7 cells/mL) were harvested by centrifugation (450 xg, 3 min). The cell pellet were washed once with BBM (Section 3.1.4.A) and resuspended in 2 mL H_2O. Then, 80 µL cell suspension were transferred with 5 µg plasmid DNA in a pre-cooled electroporation cuvette (1 mm electrode distance) and incubated for 5 min on ice. The electric shock were performed in a MicroPulser™ Electroporator (BioRad; NEU) with a capacitance at 25 µF, a resistance of 200 Ω and an electric pulse of 1500 V/cm, 1800 V/cm, or 2000 V/cm. After electroporation, the approaches were stored for 2 min on ice before they were transferred to a 15 mL reaction tube containing 2 mL BBM. These approaches were incubated on a shaker at 250 rpm and 24 °C for two hours in the dark and were then spread on BBM-plates (Section 3.1.4.A).

3.2.5.8. Agrobacterium-mediated Transformation of C. vulgaris

The *Agrobacterium* culture were freshly grown in YEP-media up to a density of OD_{600} 0.8 - 1.2. Then, 800 µL were harvested by centrifugation (12,000 xg, 30 s), washed twice with 500 µl and 100 µL *induction* media (Section 3.1.2., Table 3.4.) and added to the microalgae lawn. The microalgae lawn was obtained by first grow *C. vulgaris* in BBM for 4 days, harvest 1.5 mL (equals about 10^7 cells; 3,000 xg, 3 min), resuspend algal pellet in 100 µL BBM and plate them on co-cultivation media plates (Section 3.1.2., Table 3.4.). Here, they grew for 2 days at light (16:8 photo period) before 100 µL of the *Agrobacterium*-suspension were added and spread with glass-beads. The approaches were co-cultivated at 25 °C for 3 days in the dark. To remove the *Agrobacterium* the cells were resuspended in 1 mL BBM supplemented with timentin (100 µg/mL). The algal-*Agrobacterium* suspension were removed with the pipette and transferred into a new tube. This step was repeated until about 3 mL were collected. The suspension were incubated in a shaking incubator (200 rpm) with no direct illumination for two days. For selection of the transgenic cells, the approaches were centrifuged at 10,000 xg for 1 min. Then, the pellet were resuspended in 400 µL BBM and plated on two BBM-plates supplemented with 5 µg/mL hygB. Remaining cells were directly used for GUS-assay.

3.2.6. Staining Protocols

In this section, staining protocols for visualization of DNA in agarose gels, *gus* expression after transformation and lipid bodies in yeast cells are described.

3.2.6.1. DNA Visualization

For visualizing DNA in an agarose gel (Section 3.2.3.4.), we used RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea) at LUH. Therefore, the stain were directly added to the gel prior to pouring. At NEU, we used ethidium bromide (EtBr; Section 3.1.2., Table 3.4.). After gel electrophoresis, the gel was incubated for 5 - 15 min in a EtBr-bath before documentation.

3.2.6.2. GUS-Assay

N. benthamiana seedlings were transferred into a 50 mL reaction tube and incubated with 90 % v/v acetone for 20 min. After washing the seedlings several times with H_2O, they were immersed in fresh *GUS-staining* solution (Section 3.1.2., Table 3.4.) and incubated for 16 hours in the dark at 37 °C. Then, the seedlings were incubated in 70 % v/v ethanol for 1 - 3 days.

C. reinhardtii cells were harvested by centrifugation at 3,000 xg for 3 min, resuspended in pre-cooled 90 % v/v acetone and incubated for 20 min on ice. Then, the cells were washed twice with H_2O and resuspended in fresh *GUS-staining* solution (Section 3.1.2., Table 3.4.). The approaches were incubated for 16 hours in the dark. Then, the cells were centrifuged at 5,000 xg for 3 min, resuspended in 500 µL 70 % v/v ethanol and stored in the dark until evaluation.

3.2.6.3. Bodipy® 493/503

To stain non-polar lipids in yeast cells, the dye *Bodipy® 493/503* (Life Technologies, USA) were applied. The evaluation of the lipid bodies were performed by fluorescent microscopy or flow cytometry (Section 3.2.7.). Therefore, 10 µg / mL Bodipy® 493/503 were added to the yeast cultures. For flow cytometry the stain was incubated for 5 min before measurement (Section 3.2.7.). For microscopy, the incubation time was at least 5 min, but did not exceed 30 min.

3.2.7. Determination of Relative Lipid Contents by Flow Cytometry

The flow cytometry measurements were performed with the Cell Lab Quanta™ SC (Beckman Coulter Inc., Fullerton, USA). Therefore, *S. cerevisiae* cells were grown for 53 hours (late stationary phase, OD_{600} = 1.1 – 1.3), diluted 1:20 in PBS (Section 3.1.2., Table 3.4) and stained with *Bodipy® 493/503* 5 min prior to measurement (Section 3.2.6.3.). The decisive settings for the Cell Lab Quanta™ SC were chosen as follow: the total cell count (TCC) were set as trigger box, jumper were set to small, the photomultiplier (PMT) of the first fluorescence detector (FL1; detects light of wavelengths 500 - 550 nm) was set to 4.5, electric volume (EV-) gain were set to 4.88 and the lower level discriminator (LLD) were set to 8, the remaining settings were left in default mode. The data set of each measurement were saved in a file.

The analysis and evaluation of the raw flow cytometry data were performed with the software FlowJo vX.0.7. With this, the histograms of the FL1 detector were altered for calculation of the fluorescence mean. To avoid background fluorescence in the calculated mean, values were excluded, when its fluorescence was detected less than 20-times (in detail in Appendix, Supplementary Material 3.II). The new fluorescence mean of the data set was calculated by the program.

For the final evaluation of the flow cytometry data from one experiment, the average fluorescent mean (from three repetitions) from the positive and negative control was set to 100 % and 0 %, respectively. For calculation of the relative oil-content of the samples, their average fluorescent mean were normalized with respect to the positive and negative control. The error bars for all conditions were calculated with the highest and lowest fluorescent value obtained in the three replications with each in

respect to the positive (100 %) and negative control (0 %). The formulas used for calculation are listed below. The final values and deviations, of four independent experiments, were compiled by applying the same formulas. An example calculation is given in the Appendix (Supplementary Material 3.II).

- Appoint positive control to 100 % and negative control to 0 %:

$$\frac{{}_{mean}C^{+} - {}_{mean}C^{-}}{100} = val_{1\%}$$

- Sample calculation with respect to the controls:

$$\frac{{}_{mean}S - {}_{mean}C^{-}}{val_{1\%}} = L_{\text{int}}\%$$

- Deviation calculations with respect to the controls:
  - → upper deviation:

$$\frac{\uparrow val - {}_{mean}C^{-}}{val_{1\%}} = \uparrow_{dev}$$

  - → lower deviation:

$$\frac{\downarrow val - {}_{mean}C^{-}}{val_{1\%}} = \downarrow_{dev}$$

${}_{mean}C^{+}$	fluorescence mean from three replicated measurements from the positive control
${}_{mean}C^{-}$	fluorescence mean from three replicated measurements from the negative control
${}_{mean}S$	fluorescence mean value from three replicated measurements from a sample
$val_{1\%}$	value which equals 1 % in respect to the controls (100 % & 0 %)
$L_{int}\%$	relative light intensity of the samples in %, with respect to the controls (100 % & 0 %)
$\uparrow val$	highest measured light intensity value from three replicates
$\downarrow val$	lowest measured light intensity value from three replicates
$\uparrow_{dev}$	upper deviation from three replicates, with respect to the controls (100 % & 0 %)
$\downarrow_{dev}$	lower deviation from three replicates, with respect to the controls (100 % & 0 %)

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4. Identification and Characterization of Diacylgylcerol Acyltransferase Type-1 from *C. vulgaris*

The unsequenced green microalgae *Chlorella vulgaris* has been identified as a high oil-producing species by the Aquatic Species Program (ASP). Furthermore, it exhibits a desirable biodiesel profile containing mainly saturated and monounsaturated fatty acids. These properties make *C. vulgaris* a promising candidate for biodiesel production. In this study, we sequenced and characterized the diacylglycerol acyltransferase type-1 from *C. vulgaris* (*Cvudgat1*), which catalyzes the last step in triacylglycerol (TAG-) production and represents potentially a bottleneck. The CvuDGAT1 protein (460 aa) shares many significant motifs and several typical characteristics with other DGAT1 sequences in microalgae and plants, such as the binding domains for the two substrates acyl-CoA and diacylglycerol (DAG), the potential active site, or the highly conserved fatty acid motif. In addition, we report the first putative tertiary structure of DGAT1 and its embedding into the ER membrane. We also functionally characterized *Cvudgat1* by expressing it heterologously in a quadruple disrupted non-oil-producing yeast strain. The expression of *Cvudgat1* restored TAG-production in the yeast mutant.

4.1. Introduction

Triacylglycerols (TAGs) are among the most energy-rich and abundant forms of reduced carbon in living organisms (Ruiz-Gutierrez *et al.* 1992). TAG molecules can be converted to biodiesel through transesterification with methanol or ethanol using basic or acidic catalysis (Fukuda *et al.* 2001; Chisti 2007). Biodiesel is chemically similar to conventional diesel, but have additional advantages over fossil fuels. For example, the primary feedstocks of biodiesel are oils from renewable sources, such as that found in oil seeds and oleaginous algae (Durrett *et al.* 2008) and its production is potentially carbon neutral. Furthermore, biodiesel has a higher flashpoint than conventional diesel, which makes it easier to handle and to store in high volumes (Yu *et al.* 2011).

In plants, the biosynthesis of free fatty acids (FAs) and TAGs occur in chloroplasts and the endoplasmic reticulum (ER), respectively (Joyard *et al.* 2010; Chapter 2, Section 2.5., Figure 2.3). The first committed step of the FA pathway is catalyzed by the enzyme acetyl-CoA-carboxylase (ACCase), which converts acetyl-CoA to malonyl-CoA by an irreversible carboxylation. Upon transfer to an acyl carrier protein (ACP), malonyl-ACP enters the FA pathway, condenses with another acyl-ACP, and elongates through the action of several enzymes of the FA complex. In each cycle, two carbons are added to the ACP-bound FA chain, eventually forming C16:0-ACP. In the final step, C16:0-ACP can elongate and undergo desaturation to form C18:0-ACP and C18:1-ACP, respectively (Lung and Weselake 2006). Those FAs exit the chloroplast and are incorporated into TAGs through the *Kennedy pathway* in the ER (Kennedy 1961). The glycerol backbone of the TAG molecule is derived from *sn*-glycerol-3-phosphate (G3P). Acyltransferases sequentially attach three FA chains to glycerol to form

TAG. The key enzymes of the *Kennedy pathway* are *sn*-glycerol-3-phosphate acyltransferase (GPAT), lyso-phosphatidic acid acyltransferase (LPAAT), and diacylglycerol acyltransferase (DGAT; Ohlrogge and Browse 1995). TAG molecules accumulate in lipid bodies coated with oleosin and released into the cytoplasm (Lung and Weselake 2006). An alternative acyl-CoA-independent pathway for TAG-production has been identified in plants and yeast (Dahlqvist *et al.* 2000). In this pathway, phospholipid diacylglycerol acyltransferase (PDAT) catalyzes the transfer of the acyl units from phosphatidylcholines (PC; instead of acyl-CoA) to diacylglycerol (DAG) to form TAGs (Lung and Weselake 2006; Dahlqvist *et al.* 2000).

The DGAT enzyme catalyzes the last step in TAG-biosynthesis and has been identified as one of the key enzymes of TAG-production. Significant TAG accumulation in microalgae has been correlated with DGAT levels (Guarnieri *et al.* 2011; Miller *et al.* 2010). In some oil-producing plants, DGAT has been identified as one of the rate-limiting enzymes for oil accumulation (Li *et al.* 2013; Lung and Weselake 2006; Jako *et al.* 2001; Perry *et al.* 1999; Zou *et al.* 1999; Ohlrogge and Jaworski 1997; Ichihara *et al.* 1988). Three types of DGAT have been identified so far, DGAT1 to DGAT3. DGAT1 and DGAT2 types are integrated in the ER membrane but share no sequence homologies (La Russa *et al.* 2012; Lung and Weselake 2006). The third type DGAT (DGAT3) is a soluble cytoplasmic enzyme, which seems to play a minor role in TAG-production (Lung and Weselake 2006) and its precise function is unknown (Saha *et al.* 2006).
DGAT1 and DGAT2 isoenzymes significantly increase TAG-production (i. e. yeast mutants) but its specificity is tissue- and species-dependent. In fact, DGAT1 plays a more significant role in some plants, while DGAT2 in other plants (Shockey *et al.* 2006).

In this study, we sequenced and investigated *dgat1* from *Chlorella vulgaris* (*Cvudgat1*). The total oil-content of *C. vulgaris* can be increased to 40 - 60 % dry cell weight (DCW) oil by altering abiotic (temperature, pH) or biotic factors (i. e. nutrient limitation) or by changing from phototrophic to mixotrophic conditions (Al-Iwayzy *et al.* 2014; Praveenkumar *et al.* 2012; Mallick *et al.* 2012; Mujtaba *et al.* 2012; Kurt 2011; Yeh and Chang 2011; Guarnieri *et al.* 2011; Mata *et al.* 2010; Converti *et al.* 2009; Liu *et al.* 2008; Illman *et al.* 2000). The TAG profile of *C. vulgaris* contains mainly saturated fatty acids (C16:0 and C:18:0; Mallick *et al.* 2012; Rasoul-Amini *et al.* 2011) and a high amount of monounsaturated oleic acid (C:18:1 Δ^9; Hobuss *et al.* 2011; Guarnieri *et al.* 2011; Kurt 2011), which lowers the freezing point of its biodiesel (Ramos *et al.* 2009). With this fatty acid methyl ester (FAME) profile, *C. vulgaris* biodiesel matches the physical properties (density, viscosity, acid value, iodine value, calorific value, cetane index, ash, and water contents) required for the international ASTM (EN 14 214) and Indian (IS 15 607) biodiesel standards (Al-Iwayzy *et al.* 2014; Mallick *et al.* 2012; Rasoul-Amini *et al.* 2011).

DGAT1 is a potential bottleneck of TAG-biosynthesis and a promising target for engineering increased TAG-biosynthesis in microalgae (Ohlrogge and Jaworski 1997; Miller *et al.* 2010; Guarnieri *et al.* 2011). In the microalgae *Chlamydomonas reinhardtii,* the expression of DGAT1 was completely

suppressed under nitrogen-abundant conditions and significantly increased with high lipid accumulation under nitrogen-deprivation (Miller *et al.* 2010). Similarly, in *C. vulgaris*, the expression of all proteins in the *Kennedy pathway* increased under nitrogen depletion, especially the DGAT enzyme (Guarnieri *et al.* 2011). Based on nucleotide sequence homology to other plants or microalgae, this DGAT is the DGAT type-1. So far, DGATs from three microalgae have been sequenced and functionally characterized (Gong *et al.* 2013; La Russa *et al.* 2012; Guihéneuf *et al.* 2011; Wagner *et al.* 2010), but DGAT type-1 just in the diatom *Phaeodactylum tricornutum* (Guihéneuf *et al.* 2011).

In this study, we identified and characterized the *C. vulgaris dgat1* gene (*Cvudgat1*) using bioinformatic tools and through functional heterologous expression in yeast mutants. By applying bioinformatic tools, the localization, transmembrane domains (TMDs), and tertiary structure of DGAT1 were determined. Additionally, we investigated the impact of *Cvudgat1* heterologous expression on TAG-production by transforming the quadruple disrupted non-oil-producing yeast mutant (H1246) with the identified *Cvudgat1* gene from *C. vulgaris*.

4.2. Material and Methods

4.2.1. Maintenance of *C. vulgaris* Cultures

C. vulgaris UTEX 259 was obtained from the Culture Collection of Algae at the University of Texas at Austin. *C. vulgaris* cells were cultivated in Bold´s Basal Media (BBM; Bold 1949), modified by (Starr and Zeikus 1993) supplemented with the vitamins biotin (0.125 mg/L), cyanocobalamin (0.125 mg/L), and thiamin-HCl (0.152 mg/L). The exact culture conditions can be found in Chapter 3 Section 3.2.1.1.

4.2.2. Identification and Cloning of *dgat1* from *C. vulgaris* (*Cvudgat1*)

To obtain mRNA from *C. vulgaris*, cultures (50 mL) were grown under nutrient deprivation (half strength BBM for 7 days), harvested by centrifugation (2,000 xg, 3 min), flash-frozen in liquid nitrogen, ground to powder with mortar and pestle, and extracted for mRNA using RNAzol® RT (Molecular Research Center). The SMARTer™ RACE cDNA Amplification Kit (Clontech) was used to synthesize the cDNA and prepare the 5´- end and 3´- end cDNA libraries. The gDNA libraries were prepared by gDNA isolation from *C. vulgaris* cultures (50 mL) with the CTAB method (Chapter 3, Section 3.2.2.2.), followed the application of the GenomeWalker™ Universal Kit (Clontech, USA).

To design the primers for amplifying the *Cvudgat1* gene, the DGAT1 protein sequence from four higher plants and the microalga *Chlorella variabilis* were aligned using Clustal Omega 1.2.1. (Sievers *et al.* 2011). Highly conserved regions of the DGAT1 protein were identified (Appendix, Supplementary Material 4.I). In total four primers were derived using the corresponding nucleotide sequence of the hypothetical DGAT1 protein from the closely related *C. variabilis* (1,341 bp; 446 aa, JGIDB: ChlNC64A_1_28812). These primers (CvaCR**1**DGAT1 to CvaCR**4**DGAT1; Appendix, Supplementary Material 4.II; Chapter 3, Section 3.1.8., Table 3.13) were used to amplify parts of the *Cvudgat1* gene in a nested-PCR using the Phusion® Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific).

Then, *Cvudgat1* specific primers were derived from the obtained partial sequence (Cvudgat1**m**_f and Cvudgat1**m**_r; Chapter 3, Section 3.1.8., Table 3.13). Together with adapter primers provided in the SMARTer™ RACE cDNA Amplification Kit and the cDNA-libraries as template, the 5´- end (915 bp) and 3´- end (960 bp) were amplified with an overlapping region of 492 bp. The entire sequence was obtained in an overlapping PCR reaction with *Cvudgat1* flanking primers (Cvudgat1_f and Cvudgat1_r; Chapter 3, Section 3.1.8., Table 3.13). To obtain the gDNA of *Cvudagat1*, the primers Cvudgat1_f and Cvudgat1_r (Chapter 3, Section 3.1.8., Table 3.13) were used in a PCR with the gDNA libraries. Both *Cvudgat1* gene sequences (gDNA and cDNA) were inserted into pJET1.2 vector provided in CloneJET PCR Cloning Kit (Thermo Scientific; pJET1.2_*Cvudgat1*) and confirmed by sequencing.

4.2.3. Structural Characterization of CvuDGAT1

The evolutionary distances of CvuDGAT1 to other related DGAT1 proteins were illustrated using phylogenetic analysis compiled with the software package MEGA 6 (Tamura *et al.* 2013). First, DGAT1 sequences from plants and lower eukaryotes (listed in Figure 2) were aligned using the MUSCLE algorithm (Edgar 2004). MUSCLE alignment was performed with the following parameters: gap open penalty -2.9 and no gap extension penalty, a hydrophobicity multiplier of 1.2. UPGMA was used in each step as the clustering method. The evolutionary model and parameters appropriate for phylogeny was determined using the module “Models” from MEGA 6. Due to a BIC (Bayesian Information Criterion) of 22947 and an AICc (Akaike Information Criterion, corrected) of 22337 we have chosen JTT+G+I+F (JTT: Jones-Taylor-Thornton model) as the evolutionary model for compiling the phylogenetic tree. Robustness of tree topology was measured by testing the phylogeny with 500 bootstrap replications.

The hydrophobic regions of CvuDGAT1, which likely contain the membrane-spanning regions (transmembrane domains, TMDs), were identified using several tools: dense alignment surface (DAS, Cserzo *et al.* 1997), TMpred from the ExPASy server (Bioinformatics Resource Portal, Hofmann and Stoffel 1993), Kyte & Doolittle hydropathy plot (KD, Kyte and Doolittle 1982), Phobius (Stockholm Bioinformatics Center, Käll *et al.* 2007), SPLIT4.0 (Juretic *et al.* 2002), and TMHMM2.0 from the Center for Biological Sequence Analysis (Technical University of Denmark). Their results were compared to obtain a putative integration of DGAT1 into the ER membrane. Additionally, TMpred, Phobius, and TMHMM2.0 provided a putative orientation of CvuDGAT1 within the membrane.

The tertiary structure of the DGAT1 protein was developed with two three-dimensional protein prediction tools, which is I-TASSER[1] (Roy *et al.* 2010) and its underlying server LOMETS[2] (Wu and Zhang 2007). All modeling approaches were performed with default settings. The best models obtained from the two different servers were aligned against each other using TMalign[3] (Zhang and Skolnick 2005). Representations of all models were performed using the software package Pymol[4] (Schrödinger 2014).

1 http://zhanglab.ccmb.med.umich.edu/I-TASSER/ (09/15/2014)
2 http://zhanglab.ccmb.med.umich.edu/LOMETS/ (09/15/2014)
3 http://zhanglab.ccmb.med.umich.edu/TM-align/ (09/15/2014)
4 http://pymol.org/ (09/15/2014)

4.2.4. Functional Characterization of CvuDGAT1 in Yeast Mutants

The *Cvudgat1* gene was cloned in the yeast plasmid pYES2, kindly provided by Prof. Christoph Benning (Department of Biochemistry and Molecular Biology, Michigan State University, USA; Sanjaya *et al.* 2013). The pYES2 plasmid contains a galactose-inducible hybrid promoter (GAL1) regulating the expression of the *gene of interest* (*goi*), ampicillin resistance for bacterial selection, and the yeast gene *URA3* for selection of auxotrophic yeast mutants.

The yeast strains SCY62, H1262, and H1246 were kindly provided by Prof. Sten Stymme (Swedish University of Agricultural Science, Sundsvägen, Sweden; Sandager *et al.* 2001; Table 4.1). The H1246 strain is a quadruple disrupted yeast mutant lacking the four essential enzymes of the oil-producing pathway: DGAT1, PDAT (phospholipid diacylglycerol acyltransferase), ASAT1 (acyl-CoA sterol acyltransferase type 1), and ASAT2 (acyl-CoA sterol acyltransferase type 2; Table 4.2). The H1246 strain has lost the capability to accumulate oil, serving as the negative control, while the SCY62 strain is the *Saccharomyces cerevisiae* wild type, serving as the positive control for the experiment. The third strain H1262 lacks all genes except the *dga1* gene, serving as the direct DGAT1 control (Table 4.2). The quadruple disrupted yeast mutant H1246 was transformed with our *Cvudgat1* gene (pYES2_*Cvudgat1*) using the lithium acetate method (Gietz and Woods 2002; Chapter 3, Section 3.2.5.3.) to assess the activity of the CvuDGAT1 protein.

Table 4.1 | Yeast strains used in this study and their relevant genotypes.

Strain	Relevant genotypes
SCY62	*MAT**a** ADE2*
H1262	*MATα are1-Δ :: HIS3 are2-Δ :: LEU2 lro1-Δ :: URA3 ADE2*
H1246	*MATα are1-Δ :: HIS3 are2-Δ :: LEU2 dga1-Δ :: KanMX4 lro1-Δ :: TRP1 ADE2*

The corresponding knockouts are additionally listed and explained in Table 4.2.

Table 4.2 | Yeast strains used in this study and their lacking genes.

Strain		*dga1*	*pdat*	*asat1*	*asat2*
SCY62	- wild type	+	+	+	+
H1262	- DGAT1 positive control	+	-	-	-
H1246	- negative control	-	-	-	-

The yeast strains and their relevant genotypes are listed in Table 4.1. The genes *dga1*, *pdat*, *asat1*, and *asat2* encode the proteins DGAT1, PDAT (phospholipid diacylglycerol acyltransferase), ASAT1 (acyl-CoA sterol acyltransferase type 1) and ASAT2 (acyl-CoA sterol acyltransferase type 2), respectively. The plus indicates that the strain contains the corresponding enzyme, a minus indicates that the gene is lacking.

For the functional characterization of the CvuDGAT1 protein, the yeast strains (SCY62, H1246, and H1262) were pre-cultured in Dropout Base media (DOB) supplemented with L-histidine, L-leucine, L-tryptophan, L-uracil (MP Biomedicals), and 2 % w/v raffinose (10 ml final volume). The yeast clones (H1246 containing the pYES2_*Cvudgat1* plasmid) were pre-cultured in DOB media supplemented with L-histidine, L-leucine, L-tryptophan, and 2 % w/v raffinose but lacking uracil (selection marker). All cultures were grown at 30 °C and 220 rpm for 24 hours. After 24 hours, fresh corresponding DOB

media containing 2 % w/v galactose (in place of raffinose to induce *Cvudgat1* expression in the yeast clones) was inoculated with the pre-cultures to a final OD_{600} of 0.2 and incubated at 30 °C and 220 rpm for 53 hours.

To evaluate the relative oil-content, cultures were diluted 1:20 in PBS and stained with the lipophilic dye Bodipy® 493/503 (final concentration of 10 µg / mL for 5 min; Life Technologies). The oil-content of the stained yeast strains was visualized by fluorescence microscope (Nikon ECLIPSE 80i, camera: SPOT Insight™ Camera 2.0 Mp Color). Additionally, the fluorescence of the stained yeast cells was quantified by flow cytometry (Cell Lab Quanta™ SC, Beckman Coulter®). Four independent experiments with triplicate measurements for each yeast strain were performed. For evaluation of the relative oil-content measured by flow cytometry, the DGAT1 positive control and the *Cvudgat1* expressing clones were normalized with respect to the wild type control (100 %) and the negative control (non-oil-producing quadruple disrupted H1246 line; 0 %; Chapter 3, Section 3.7.2.).

4.3. Results and Discussion

4.3.1. Sequencing and Phylogenetic Comparison of CvuDGAT1

To obtain the *dgat1* gene sequence from *C. vulgaris* (*Cvudgat1*), we first aligned the DGAT1 protein sequences of the microalga *C. variabilis* and higher plants to identify highly conserved regions (Appendix, Supplementary Material 4.I). The cDNA sequence encoding these highly conserved regions were obtained from the closely related *C. variabilis* and served as the primers for amplifying the *dgat1* gene from the *C. vulgaris* cDNA library (Appendix, Supplementary Material 4.II; Chapter 3, Section 3.1.8., Table 3.13). The assembled *Cvudgat1* cDNA sequence is 1,383 bp (Appendix, Supplementary Material 4.IV). By applying the gDNA library with the flanking *Cvudgat1* primers, we obtained the complete gDNA sequence of the *Cvudgat1* gene with 2,854 bp (Appendix, Supplementary Material 4.III). The protein sequence (CvuDGAT1) has 460 amino acids (MW 53.5 kDa; Appendix, Supplementary Material 4.V).

We compared the *C. vulgaris* DGAT1 protein sequence to 31 other related DGAT1s in a phylogenetic tree using MEGA 6 (Tamura *et al.* 2013). The results are summarized in Figure 4.1. The phylogenetic analysis was performed using the maximum likelihood method based on the JTT matrix-based model (Jones *et al.* 1992; Section 4.2.3.). Here, the tree with the best log likelihood is shown. The tree is drawn to scale and shows the relative distances, with branch lengths measured in the number of substitutions per site. Evolutionary analysis were conducted in MEGA 6 (Tamura *et al.* 2013). The scale bar represents relative divergence times of branches only.

The amino acid homologies of other DGAT1s to CvuDGAT1 were compiled with Clustal W2.1 (Larkin *et al.* 2007). We found that the DGAT1 protein sequence of the related species *C. variabilis* (CvaDGAT1) and CvuDGAT1 share the highest homology of 79 %, as expected. In comparison to all applied DGAT1 sequences from green algae, *Chlamydomonas reinhardtii* (CrDGAT1) has the lowest homology to CvuDGAT1 with 49 %. The CvuDGAT1 shares higher sequence homology with certain plants (*Oryza sativa*, OsDGAT1, 42 %; *Euonymus alatus*, EaDGAT1, 46 %) than with diatoms (*Thalassiosira pseudonana*, TpDGAT1, 38 %; *Phaeodactylum tricornutum*, PtDGAT1, 41 %).

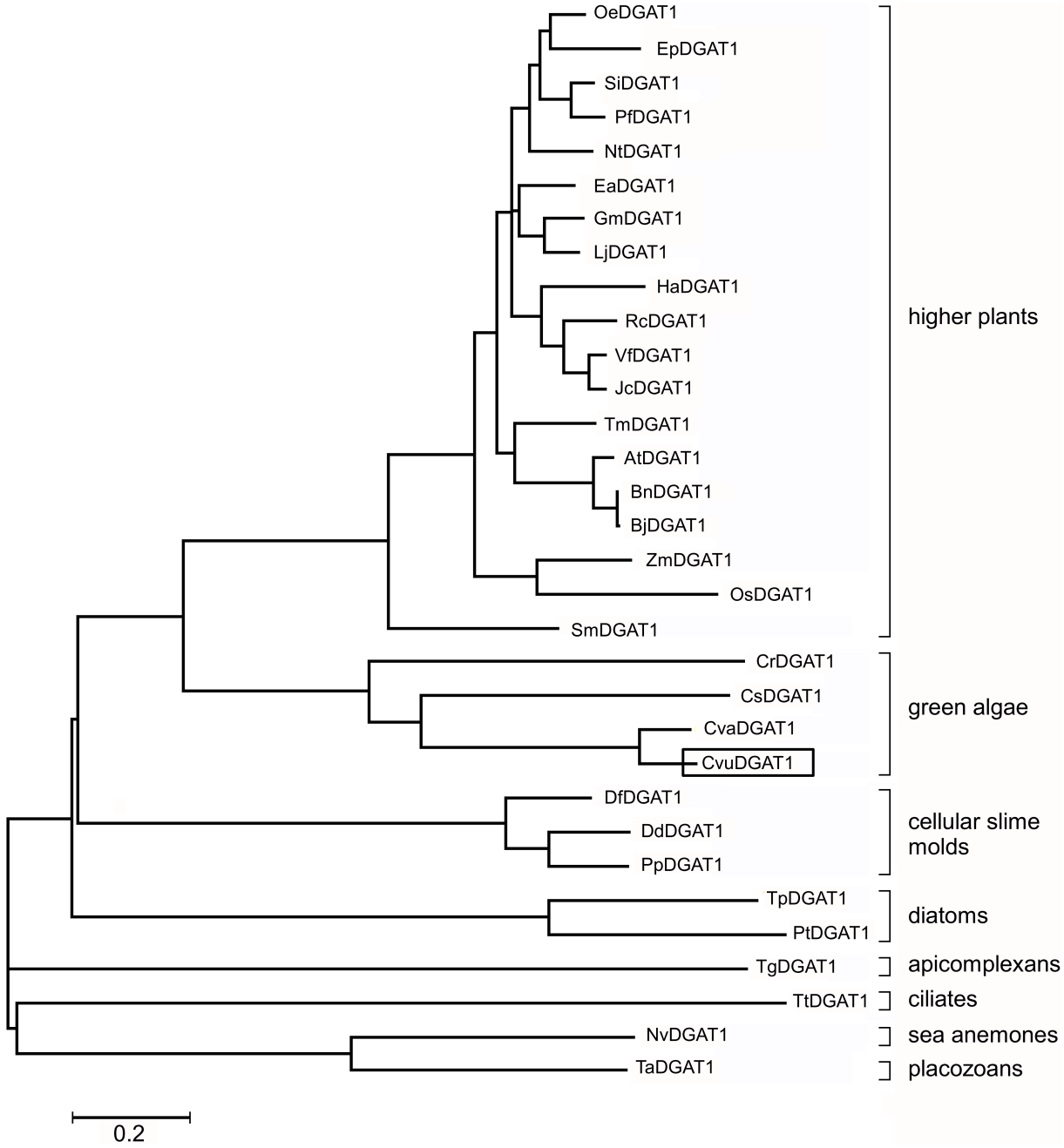


Figure 4.1 | Phylogenetic tree of diverse DGAT1 protein sequences shows the relationships among *C. vulgaris* DGAT1 (CvuDGAT1) and several other characterized DGAT1 proteins from higher plants, green algae, and diatoms. Also hypothetical DGAT1 sequences from cellular slime molds, apicomplexans, ciliates, sea anemones, and placozoans were used for the phylogenetic assignment of CvuDGAT1. The phylogram was compiled with MEGA 6 (Tamura *et al.* 2013). The following species were used for the phylogenetic tree: **Placozoans**: *Trichoplax adhaerens* (TaDGAT1, XP_002112025.1); **sea anemones**: *Nematostella vectensis* (NvDGAT1, XP_001639351.1); **ciliates**: *Tetrahymena thermophila* (TtDGAT1, XP_001014621.2); apicomplexans: *Toxoplasma gondii* (TgDGAT1, AAP94209.1); **diatoms:** *Thalassiosira pseudonana* CCMP1335 (TpDGAT1, XP_002287215.1), *Phaeodactylum tricornutum* (PtDGAT1, ADY76581.1); **cellular slime molds**: *Dictyostelium fasciculatum* (DfDGAT1, XP_004366137.1), *Dictyostelium discoideum* AX4 (DdDGAT1, XP_645633.2), and *Polysphondylium pallidum* PN500 (PpDGAT1, EFA85004.1); **green algae**: *Chlamydomonas reinhardtii* (CrDGAT1, (Boyle *et al.* 2012), *Coccomyxa subellipsoidea* (CsDGAT1, EIE20990.1), *Chlorella variabilis* (CvaDGAT1, EFN50697.1), *Chlorella vulgaris* (CvuDGAT1); **higher plants**: *Olea europaea* (OeDGAT1, AAS01606.1), *Echium pitardii* (EpDGAT1, ACO55635.1), *Sesamum indicum* (SiDGAT1, AEE37277.1), *Perilla frutescens* (PfDGAT1, AAG23696.1), *Nicotiana tabacum* (NtDGAT1, AAF19345.1), *Euonymus alatus* (EaDGAT1, AAV31083.1), *Glycine max* (GmDGAT1, AAS78662.1), *Lotus japonicus* (LjDGAT1, AAW51456.1), *Helianthus annuus* (HaDGAT1, ADT91687.1), *Ricinus communis* (RcDGAT1, XP002514132.1), *Vernicia fordii* (VfDGAT1, ABC94471.1), *Jatropha curcas* (JcDGAT1, ABB84383.1), *Tropaeolum majus* (TmDGAT1, AAM03340.2), *Arabidopsis thaliana* (AtDGAT1, NP179535.1), *Brassica napus* (BnDGAT1, AAD45536.1), *Brassica juncea* (BjDGAT1, AAY40784.1), *Zea mays* (ZmDGAT1, ABV91586.1), *Oryza sativa* (OsDGAT1, NP 001054869.2), *Selaginella moellendorffii* (SmDGAT1, XP 002994237.1).

4.3.2. Motif Identification in CvuDGAT1

We aligned the CvuDGAT1 protein sequence with eleven characterized DGAT1 sequences, including that of three different green algae (*C. variabilis*, CvaDGAT1; *Chlamydomonas reinhardtii*, CrDGAT1; *Coccomyxa subellipsoidea*, CsDGAT1), two diatoms (*Phaeodactylum tricornutum*, PtDGAT1; *Thalassiosira pseudonana*, TpDGAT1), and six oil-producing or model plants (*Vernicia fordii*, VfDGAT1; *Ricinus communis*, RcDGAT1; *Olea europaea*, OeDGAT1; *Glycine max,* GmDGAT1; *Arabidopsis thaliana*, AtDGAT1, and *Zea mays,* ZmDGAT1). Several conserved sequence domains have been functionally characterized in other DGAT1 proteins and were also found in CvuDGAT1 (Figure 4.2; complete alignment see Appendix, Supplementary Material 4.VI).

The N-terminal end of CvuDGAT1 is less conserved than the C-terminal end, as previously reported (Li *et al.* 2013, Guihéneuf *et al.* 2011). Generally, the length of the green algae DGAT1s is at least 60 aa shorter on the N-terminal end than the DGAT1s from higher plants (Appendix, Supplementary Material 4.VI). Hence, the green algae DGAT1 sequences do not contain the characteristic basic RRR motif found in higher plants or similarly, the KRS motif in the diatom *P. tricornutum* (PtDGAT1; Guihéneuf *et al.* 2011; Appendix, Supplementary Material 4.VI).

We identified possible binding domains for the two substrates of DGAT1, acyl-CoA and DAG. The **acyl-CoA binding site (I)** is located at the N-terminal end of the DGAT1 sequence. In higher plants, this signature is about 31 residues (AtCvuDGAT1: Arg^{116} – Val^{146}; Figure 4.2; Mañas-Fernández *et al.* 2009). In diatoms, this motif is shortened by one amino acid at position 87 and 35 for PtDGAT1 and TpDGAT1, respectively (Guihéneuf *et al.* 2011). In green algae, the acyl-CoA binding site is further shortened but not for *C. vulgaris*. We suggest the acyl-CoA binding site of CvuDGAT1 has the same domain size as that of plants with 32 residues (Ala^{2} – Ala^{32}). The **DAG/phorbol ester binding motif (IV)** is located closer to the C-terminal end of the DGAT1 protein sequence (Figure 4.2; Oelkers *et al.* 1998). This binding domain of 14 residues (Val^{346} to Leu^{359}) is more conserved than the acyl-CoA binding site with high sequence conformity also between higher plants and green algae (Guihéneuf *et al.* 2011; Mañas-Fernández *et al.* 2009).

Furthermore, we identified the **putative active site (II)** of the DGAT1 protein, located adjacent to the acyl-CoA binding site. This conserved domain contains the motif **N**(S/A/G)**R**(L/V)(I/F/A)(I/L)**E**N (Figure 4.2; Jako *et al.* 2001; whereas the F on the second position is also very likely). This motif was determined from structure function relationships to the related acyl-CoA-dependent GPAT and LPAAT. In those acyltransferases, the conserved domain X**H**XXX(X)**D** has been identified as part of the active site. Within this motif the histidine residue (H) extracts a proton from the hydroxyl group of the aspartic acid (D), which is the acyl-acceptor by facilitating a nucleophilic attack on the thioester bond of the acyl donor (acyl-CoA). The arginine in the GPAT and LPAAT proteins in turn stabilizes the positive charge on the histidine imidazole ring (Frentzen and Wolter 1998). Jako *et al.* (2001)

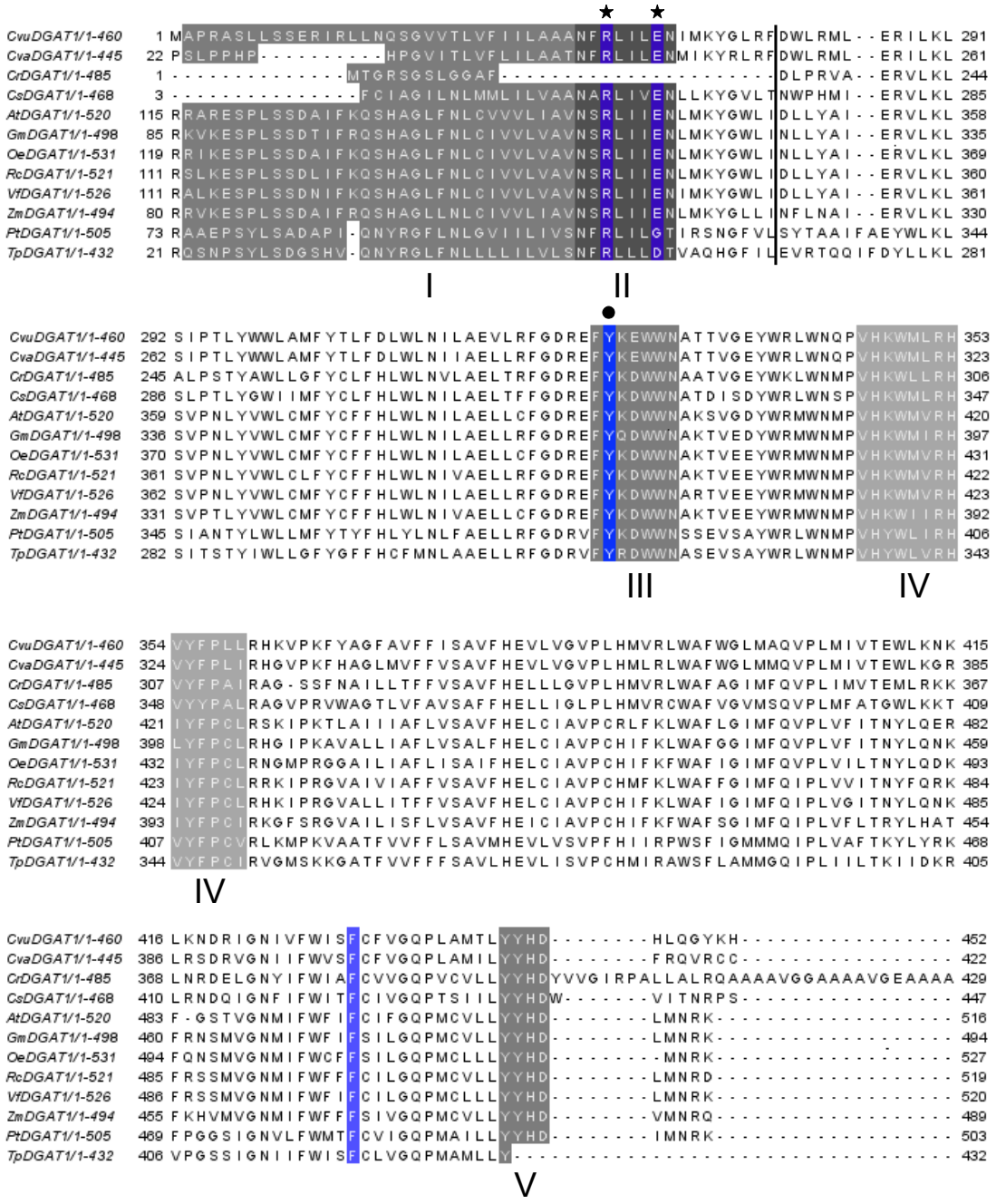


Figure 4.2 | Sequence homology comparison of CvuDGAT1 with DGAT1 protein sequences from green algae, diatoms, and higher plants. The CvuDGAT1 sequence was aligned against characterized DGAT1 amino acid sequences from green algae, plants, and diatoms with MEGA 6 (Tamura *et al.* 2013). **Green algae**: *Chlorella variabilis* (CvaDGAT1, EFN50697.1), *Chlamydomonas reinhardtii* (CrDGAT1, (Boyle *et al.* 2012) , and *Coccomyxa subellipsoidea* (CsDGAT1, EIE20990.1); Oil-producing or model **plants**: *Arabidopsis thaliana* (AtDGAT1; NP179535.1), *Glycine max* (GmDGAT1, AAS78662.1), *Olea europaea* (OeDGAT1, AAS01606.1), *Ricinus communis* (RcDGAT1, XP002514132.1), *Vernicia fordii* (VfDGAT1, ABC94471.1), *Zea mays* (ZmDGAT1, ABV91586.1); **diatoms**: *Phaeodactylum tricornutum* (PtDGAT1, ADY76581.1) and *Thalassiosira pseudonana* CCMP1335 (TpDGAT1, XP_002287215.1). Conserved motifs and putative signatures are numbered from I to V (see text for details). We identified the acyl-CoA binding motif (**I**), the putative active site of DGAT1 (**II**, black box), the highly conserved fatty acid protein signature (**III**), containing a putative tyrosine phosphorylation site (●), the DAG/phorbol ester binding motif (**IV**), and the putative retrieval motif (**V**). Within the predicted active site (II) the amino acids arginine (R; denoted by asterisk) and glutamine (E; denoted by asterisk) play important roles in the enzymatic reaction. The highly conserved amino acid phenylalanine (F) is highlighted in white with black background. One sequence part were cut out. Its position is marked with a vertical line.

suggested that the conserved arginine (R) and glutamine (E) residues in DGAT1s perform similar functions to those of the basic histidine (H) and aspartic acid (D) residues found in GPATs and LPAATs.

Other motifs that affect the activity of DGAT1 are the ancestral phenylalanine and the highly conserved fatty acid protein signature (F**Y**xDWWN; Figure 4.2). The ancestral **phenylalanine** (F, Phe^{469}) in DGAT1-2 of *Zea mays* has a high impact on the oil and oleic acid content (Zheng *et al.* 2008). For example, maize mutants without the Phe^{469} demonstrated less enzyme activity, while its re-insertion resulted in increased oil and oleic acid content by 41 % and 107 %, respectively (Zheng *et al.* 2008). The highly conserved phenylalanine has also been identified in CvuDGAT1 (Phe^{430}), other green algae, and diatoms (Figure 4.2). The conserved phenylalanine in plants, green algae, and diatoms suggests the importance of this amino acid for efficient DGAT1 enzyme activity. Next to the substrate binding sites and active sites, we also identified a highly conserved **fatty acid protein signature** (F**Y**xDWWN; **III**; CvuDGAT1: Phe^{325} – Asn^{331}), a distinguishing mark of all characterized O-acyltransferases (Turchetto-Zolet *et al.* 2011; Coleman and Lee 2004; Guo *et al.* 2001). The conserved tyrosine (Y, CvuDGAT1: Tyr^{326}) is a putative phosphorylation site and is believed to regulate DGAT1 activity (Buhman *et al.* 2001; Oelkers *et al.* 1998). The substitution of this tyrosine with alanine decreased the enzyme activity to 80 % of the wild type (Xu *et al.* 2008). In *C. vulgaris* and *C. variabilis*, the aspartic acid (D) in this motif has changed to glutamic acid (E; CvuDGAT1: Glu^{328}) caused by a single nucleotide polymorphism, which does not seem to impact DGAT1 activity.

Additionally, we identified a putative retrieval **YYHD-like motif (V)** within the C-terminal end (CvuDGAT1: Tyr^{442} – Asp^{445}), which is involved in embedding of DGAT1 into the ER membrane (Guihéneuf *et al.* 2011; Mañas-Fernández *et al.* 2009). A similar motif has also been identified in fatty acid desaturases type-2 (FAD2) enzymes, which are also integrated in the ER membrane (McCartney *et al.* 2004).

4.3.3. Bioinformatic Characterization of CvuDGAT1

CvuDGAT1 is a member of the membrane bound O-acyltransferase (**MBOAT**) superfamily. All biochemically characterized MBOAT members encode enzymes that transfer organic acids (mainly FAs) onto hydroxyl groups of membrane-embedded targets. These proteins contain usually between eight and ten TMDs (Hofmann 2000).

Localization

The localization of CvuDGAT1 was assessed using SignalP 4.1 (Nordahl Petersen *et al.* 2011). SignalP 4.1 predicts a low likelihood of a signal peptide in our CvuDGAT1 (24 %) or other green algae (CvaDGAT1, 41 % and CrDGAT1, 68 %). However, all characterized DGAT1 sequences contain the putative retrieval motif in the C-terminal end, which allows the protein to be embedded into the ER membrane (Section 4.3.2.; Guihéneuf *et al.* 2011; Mañas-Fernández *et al.* 2009). We suggest that the retrieval motif substitute the signal peptide in DGAT1 proteins. The association of CvuDGAT1 with the ER is consistent with the localization of TAG-biosynthesis in the ER.

Secondary structure predictions

The putative TMDs in CvuDGAT1 and the orientation of the N- and C-terminal end of CvuDGAT1 in the ER membrane were determined using various prediction tools (Section 4.2.3.).

In CvuDGAT1, nine strongly hydrophobic regions were predicted (Figure 4.3; Table 4.3). Surprisingly, several servers (DAS, TMpred, KD, and Phobius) predicted ten to eleven strongly hydrophobic regions in CvaDGAT1, the closest relative of *C. vulgaris* (Table 4.3). The diatom *P. tricornutum* is predicted to contain eight to ten putative TMDs, at least eight putative TMDs were reported by Guihéneuf *et al.* (2011). All investigated plant DGAT1s used in the alignment (Figure 4.2) have nine putative TMDs Table 4.3. In the literature, nine TMDs were predicted for DGAT1 of higher plants (*Tetraena mongolica*, Li *et al.* 2013; *Helianthus annuus L.*, Sun *et al.* 2011; *Vernonia galamensis*, Yu *et al.* 2008; *Ricinus communis*, He *et al.* 2004). In applying these tools, it should be considered that the prediction is performed considering the hydrophobicity of every amino acid, irrespective of the tertiary structure, and thereby is just an indicator.

The putative orientation of CvuDGAT1 was predicted using the servers Phobius, TMpred, and TMHMM based on their predicted hydrophobic regions. The N-terminal end of CvuDGAT1 is directed into the cytosol, while the C-terminal end is directed into the lumen of the ER (Figure 4.3C). This putative orientation matches that predicted for other DGAT1s with nine TMDs, including that of mice, humans, and other plants (FcFie *et al.* 2010, Joyce *et al.* 2000, Yu *et al.* 2008). However, for DGAT1s with ten TMDs, the C- and the N-terminal end are predicted to be directed into the cytosol (Shockey *et al.* 2006). We propose that the N-terminal end, which contains the putative acyl-CoA binding site, is likely directed into the cytosol to bind the substrate and catalyze the reaction while the C-terminal end is less significant and can point either in the ER lumen or cytosol.

Table 4.3 | TMD prediction of several DGAT1 sequences by using six different prediction servers.

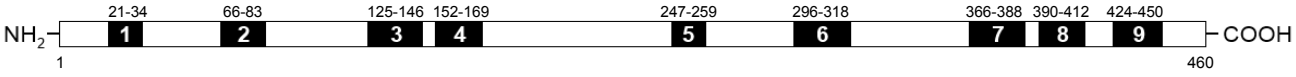
		DAS (cut off 1.7)	TMpred (Expasy)	KD Ws=9	Phobius	SPLIT4.0	TMHMM	**Conclusion**	Distribution in membrane
plants	OeDGAT1	8	10	10	9	8	9	**9 - 10**	||||-|||-|||
	VfDGAT1	9	9	9	9	8	9	**9**	||||-||-|||
	RcDGAT1	10	9 - 10	8	9	8 - 9	9	**9**	||||-||-|||
	AtDGAT1	10	10	10	9 - 10	8 - 9	9	**9**	||||-||-|||
diatoms	PtDGAT1	10	9	8	9	8 - 9	8	**9**	||||-||-|||
green algae	CrDGAT1	11	11	10	9	11	12	**11**	||-||-||-|||||
	CsDGAT1	9 - 11	10	8	8	8 - 9	9	**8 - 10**	||-||-|||-|||
	CvaDGAT1	9 - 11	11	10	10	9	9	**9 - 11**	|-|||-||-||-|||
	CvuDGAT1	9	9	9	9	8 - 9	9	**9**	||-||-||-|||

For this analysis the prediction server **DAS** (Cserzo *et al.* 1997), **TMpred** from ExPASy server (Bioinformatics Resource Portal, Hofmann & Stoffel 1993), Kyte & Doolittle hydropathy plot (**KD**, Kyte, J. and Doolittle, R. 1982), **Phobius** (Stockholm Bionformatics Center, Kaell, Krogh and Sonnhammer 2007), **SPLIT4.0** (Juretic, Zoranic and Zucic 2002), and **TMHMM2.0** from the Center for Biological Sequence Analysis (Technical University of Denmark) were used. Ind this prediction we compared the DGAT1 protein sequences from plants, a diatom, and green algae. Plants: *Olea europaea* (OeDGAT1), *Vernicia fordii* (VfDGAT1), *Ricinus communis* (RcDGAT1), and *Arabidopsis thaliana* (AtDGAT1); diatom: *Phaeodactylum tricornutum* (PtDGAT1); green algae: *Chlamydomonas reinhardtii* (CrDGAT1), *Coccomyxa subellipsoidea* (CsDGAT1), *Chlorella* variabilis (CvaDGAT1), and *Chlorella vulgaris* (CvuDGAT1). The numbers are the total amount of putative membrane-spanning regions of the DGAT1 protein. The conclusion column is the average of the predicted strong hydrophobic regions listed. The distributions of the TMDs in the ER membrane are also shown (see text for details).

A

```
001   MAPRASLLSS ERIRLLNQSG VVTLVFIILA AANFRLILEN IMKYGLRFNP FSFLKNAITP   060
061   SGNTPLVLCW PLLAFFALSA LAIERFAVRL LDMERRAAAV TDKRSVGYTE MNKAAARRAS   120
121   ATEHLVFVLN LLNTTAALLA PCAIILHTCA ELMPGFALTM ATCVLWLKLV SYAHVNFDYR   180
181   VARRRGETRP GERGSGQYPE NVEQELRYPE NIALGNLAYF LAAPTLCYQI TYPRSKRFRA   240
241   RWMVKKLFMF TGGLGLMLFV TEQYIQPTID NSMRPLKEMD WLRMLERILK LSIPTLYWWL   300
301   AMFYTLFDLW LNILAEVLRF GDREFYKEWW NATTVGEYWR LWNQPVHKWM LRHVYFPLLR   360
361   HKVPKFYAGF AVFFISAVFH EVLVGVPLHM VRLWAFWGLM AQVPLMIVTE WLKNKLKNDR   420
421   IGNIVFWISF CFVGQPLAMT LYYHDHLQGY KHPFQGTPII -                       460
```

B



C

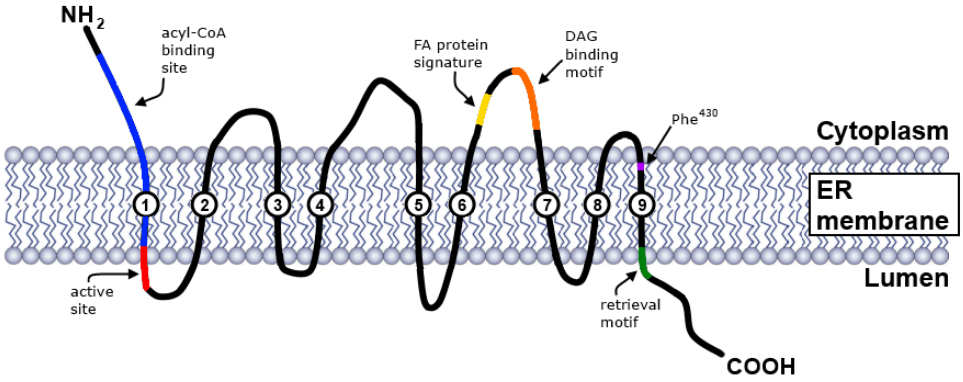


Figure 4.3 | Distribution of TMDs of the DGAT1 protein from *C. vulgaris* and its putative integration into the ER membrane. A, the amino acids of CvuDGAT1 embedded into the ER membrane are framed and highlighted in gray. **B,** shown is a consensus model of the distribution of putative TMDs of CvuDGAT1. This model is based on six TMD prediction tools (see text for details, Table 4.3). **C**, shown is the possible integration into the ER membrane. The motifs for substrate binding (acyl-CoA and DAG binding motif), activity / regulation of CvuDGAT1 (active site and phenylalanine at position 430, Phe^{430}), the FA protein signature, and the retrieval motif at the C-terminal end of the CvuDGAT1 sequence are illustrated (Figure 4.2, see text for details).

Tertiary structure of CvuDGAT1[1]

Currently, the putative tertiary structure of a DGAT1 protein is not reported. The three dimensional structure of CvuDGAT1 and the distribution of the TMDs and localization of important motifs within the three dimensional protein were predicted using two different automated prediction servers (I-TASSER, LOMETS; Section 4.2.3.).

Results from LOMETS indicated that no known homologous structure exist. Closest homologies found were an oligosaccharyl transferase from *Archaeoglobus fulgidus* (3waj) and a translocon-associated membrane protein from *Thermus thermophiles* (3AQP), both with medium confidence only. Therefore,

1 This analysis was performed by Dr. Thomas Reinard (LUH; Institute for Plant Genetics)

we decided to choose the *ab initio* automated prediction server I-TASSER (placed on rang 1 for modeling as “Zhang server” in the “10th Community Wide Experiment on the Critical Assessment of Techniques for Protein Structure Prediction” in 2012; CASP 10).

The putative tertiary structure of the CvuDGAT1 protein with the labeled TMDs and featured motifs are illustrated in Figure 4.4. Modeled CvuDGAT1 contains mainly α-helices and no ß-sheets. In the side view (Figure 4.4A), it is visible how the protein is embedded in the ER membrane and also how the N- and the C-terminal ends are directed to the cytosol. This differs significantly from the theoretical data obtained from transmembrane prediction servers (Figure 4.3; Table 4.3). A reason for this disagreement is the tendency of TMD-prediction servers to predict the TMDs just based on the hydrophobicity of every amino acid without consideration of the tertiary structure and its intramolecular interactions. The TMD-prediction servers showed that motif I and II (Figure 4.4) were integrated into the membrane rather than to be directed to the cytosol.
In consideration of the tertiary model of CvuDGAT1, the acyl-CoA binding motif is located on the cytoplasmic side of the ER membrane. The active site is located adjacent to this binding domain. Directly below the active site, but embedded in the ER membrane, is the DAG/phorbol ester binding motif is located (Figure 4.4A). This result is in agreement with the findings on other proteins containing this motif (Rahman and Das 2014). Surprisingly, both substrate binding domains are nearby in the tertiary model even though there are 314 residues of separation in the protein sequence (acyl-CoA binding domain: Ser^{10} to Ala^{32} , DAG binding domain: Val^{346} to Leu^{359} ; Figure 4.4A). In the above view of the protein from the cytosolic side (Figure 4.5B), a channel towards the DAG binding domain is visible. We suggest that the acyl-CoA chain is ushered into this channel and then attached to the DAG molecule to yield the final TAG molecule. The relevant phenylalanine residue (F; Phe^{430}), predicted to be essential for the enzyme activity (Zheng *et al.* 2008), is located on the cytosolic part of the protein, close to its catalytic side (Figure 4.4). The only motif found to be directed to the ER lumen is the fatty acid protein signature, characteristic for O-acyltransferases (Turchetto-Zolet *et al.* 2011; Coleman and Lee 2004; Guo *et al.* 2001).

In conclusion, the predicted tertiary structure of DGAT1 has a reliable overall score of confidence as determined by I-TASSER. The predicted proximity of the substrate binding domains, the active site, and the essential phenylalanine at position 430 to each other guarantees efficient substrate conversion of acyl-CoA and DAG to form TAG.

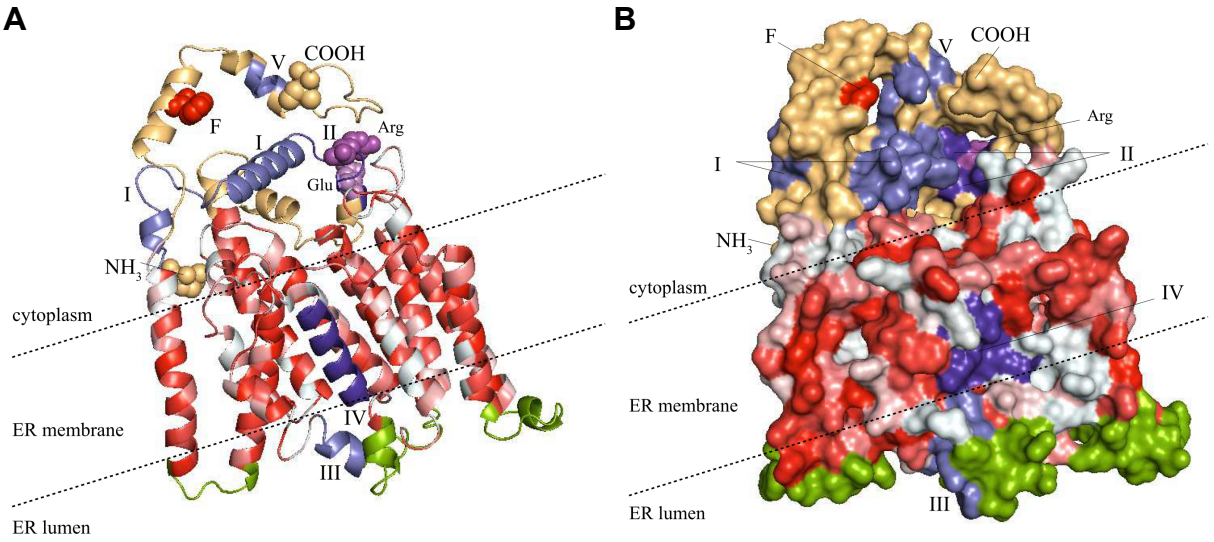


Figure 4.4 | Putative tertiary structure of CvuDGAT1. The tertiary structure of CvuDGAT1 was predicted by I-TASSER (Roy *et al.* 2010) with default settings. Representations and labeling for all models were performed with the software Pymol (Schrödinger). The putative tertiary structure of CuDGAT1 is shown in side view, as (**A**) cartoon model and (**B**) surface model. Putative TMDs are colored in red/white (pymol color_h-script) according to the Eisenberg hydrophobicity (Eisenberg *et al.* 1984). Furthermore, the most important motifs are labeled as follows: The putative binding domains for the substrates Acyl-CoA (**I**, blue) and DAG (**IV**, dark blue), the active sites within the amino acids **Arg** and **Glu**, represented as light purple spheres. Also shown as spheres are the N-terminal methionine, the carboxy-terminal isoleucine, as **NH_3** and **COOH**, respectively. The highly conserved phenylalanine at position 430 (Phe^{430}, **F**) is colored in red. This phenylalanine is essential for DGAT1 enzyme activity (see text for details). The putative retrieval motif (**V**) is located next to Phe^{430} on the cytoplasmic side of the protein.

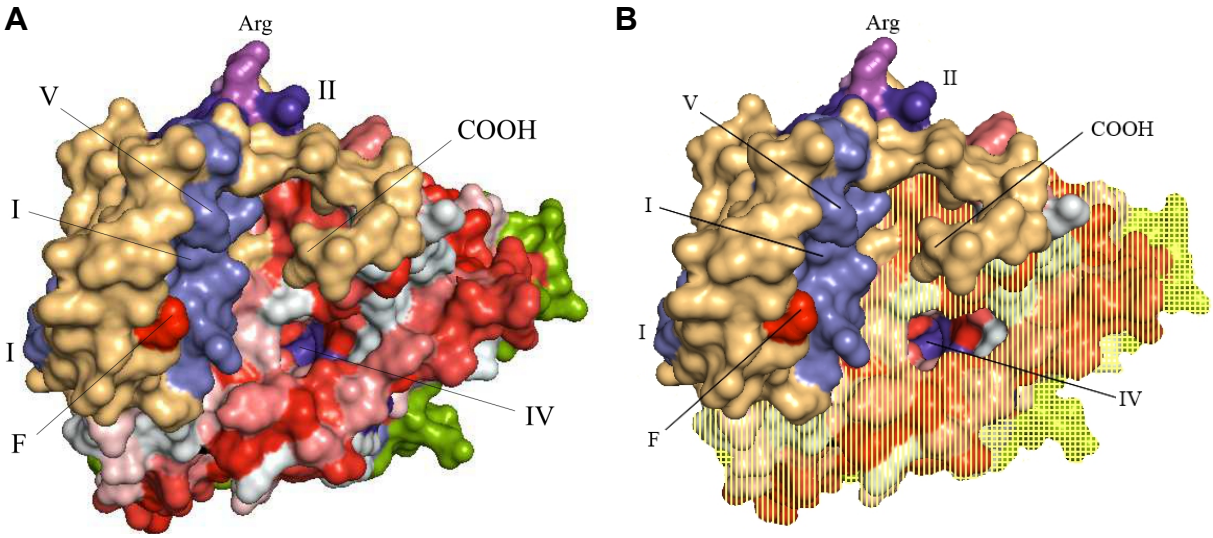


Figure 4.5 | Top view on the cytoplasmic part of the putative CvuDGAT1 protein. (**A**) unrestricted view, (**B**) restricted view to visualize how CvuDGAT1 could be integrated in the ER membrane. The CvuDGAT1 part overlayed with yellow stripes is membrane-embedded, the checked part is directed into the ER-lumen. The most important motifs are labeled as in Figure 4.4. The glutamine of the active site is directed into the protein structure and not visible. The motif III, the fatty acid protein signature, is directed to the ER lumen and from this view on the averted site of the protein.

4.3.4. Functional Characterization of CvuDGAT1 in Yeast Mutants

We functionally characterized CvuDGAT1 by expressing the protein in a yeast mutant that cannot produce TAG or steryl esters (H1246 quadruple disrupted mutant; Sandager *et al.* 2001). The H1246 quadruple disrupted mutant yields low lipid content compared to the wild type since it lacks the essential enzymes of the oil-producing pathway (Section 4.2.4., Table 4.2; Sandager *et al.* 2001). The *Cvudgat1* gene was cloned into the yeast expression plasmid pYES2 under the control of the galactose-inducible *GAL1* promoter and introduced into the yeast mutant H1246. The wild type strain (*Saccharomyces cerevisiae*, SCY62) and the H1262 mutant (lacks the same genes as H1246 except for *dgat1*; Section 4.2.4., Table 4.2) served as the positive controls, while the H1246 quadruple mutant served as negative control.

These yeast strains were first pre-cultured in promoter-suppressing conditions (uninduced condition) and then transferred to fresh media containing the inducer galactose to initiate expression of *Cvudgat1* (induced condition). Because storage lipids accumulate mainly during the late stationary phase (Sandager *et al.* 2001), we evaluated the oil-content after this growth phase was reached. The impact of the heterologous expression of *Cvudgat1* on the total lipid content was visualized using Bodipy® 493/503 and evaluated by fluorescence microscopy and flow cytometry (Figure 4.6 and 4.7).

The wild type (SCY62) and DGAT1 positive control (H1262) lines clearly exhibit fluorescently labeled lipid bodies when grown with and without the inducer galactose (Figure 4.6). As expected, no lipid bodies were detected in the negative control line (H1246, quadruple disrupted mutant) under both conditions. These results were similar to that observed by Sandager *et al.* (2001). In the absence of the inducer, the transformed H1246 strain containing the *Cvudgat1* gene (+*Cvudgat1* clones 1 and 2) did not contain lipid bodies, similar to the negative control. However, in the presence of the inducer galactose, a majority of the transformed yeast cells contained lipid bodies.

The oil-content of Bodipy® 493/503 stained yeast cells was also quantified by flow cytometry (Figure 4.7). The oil-content of the DGAT1 positive control (H1262) was 63.9 % of the wild type, similar to the levels (71 %) obtained by Sandager *et al.* (2001). The oil-content of the two heterologous expressing *Cvudgat1* clones were 49.0 % and 52.8 % to the wild type strain (calculation see Chapter 3, Section 3.2.7.). The availability of specific FAs is species-dependent and likely resulted in the lowered oil-content of the *Cvudgat1* clones compared to DGAT1 positive control (H1262). Yeast preferentially produces monounsaturated FA such as oleic acid (C18:1 Δ^9) and palmitoleic acid (C16:1 Δ^9). However, *C. vulgaris* favors oleic acid (C18:1 Δ^9) and palmitic acid (C16:0) followed by polyunsaturated FAs, such as linoleic acid (C18:2 $\Delta^{9,12}$) and α-linolenic acid (C18:3 $\Delta^{9,12,15}$; Kurt 2011), which are less available in yeast (Table 4.4).

In summary, the yeast quadruple disrupted mutant did not accumulate oil, whereas those rescued with *Cvudgat1* accumulated levels similar to the yeast mutant expressing only the yeast *dgat1* gene (DGAT1 positive control, H1262). The heterologous expression of *dgat1* from the higher plants *Tropaeolum majus* and *Arabidopsis thaliana* also restored oil-production of the quadruple disrupted yeast mutant (Xu *et al.* 2008; Bouvier-Nave *et al.* 2000). Our results confirm that the DGAT1 protein from *C. vulgaris* has functional activity in oil-production.

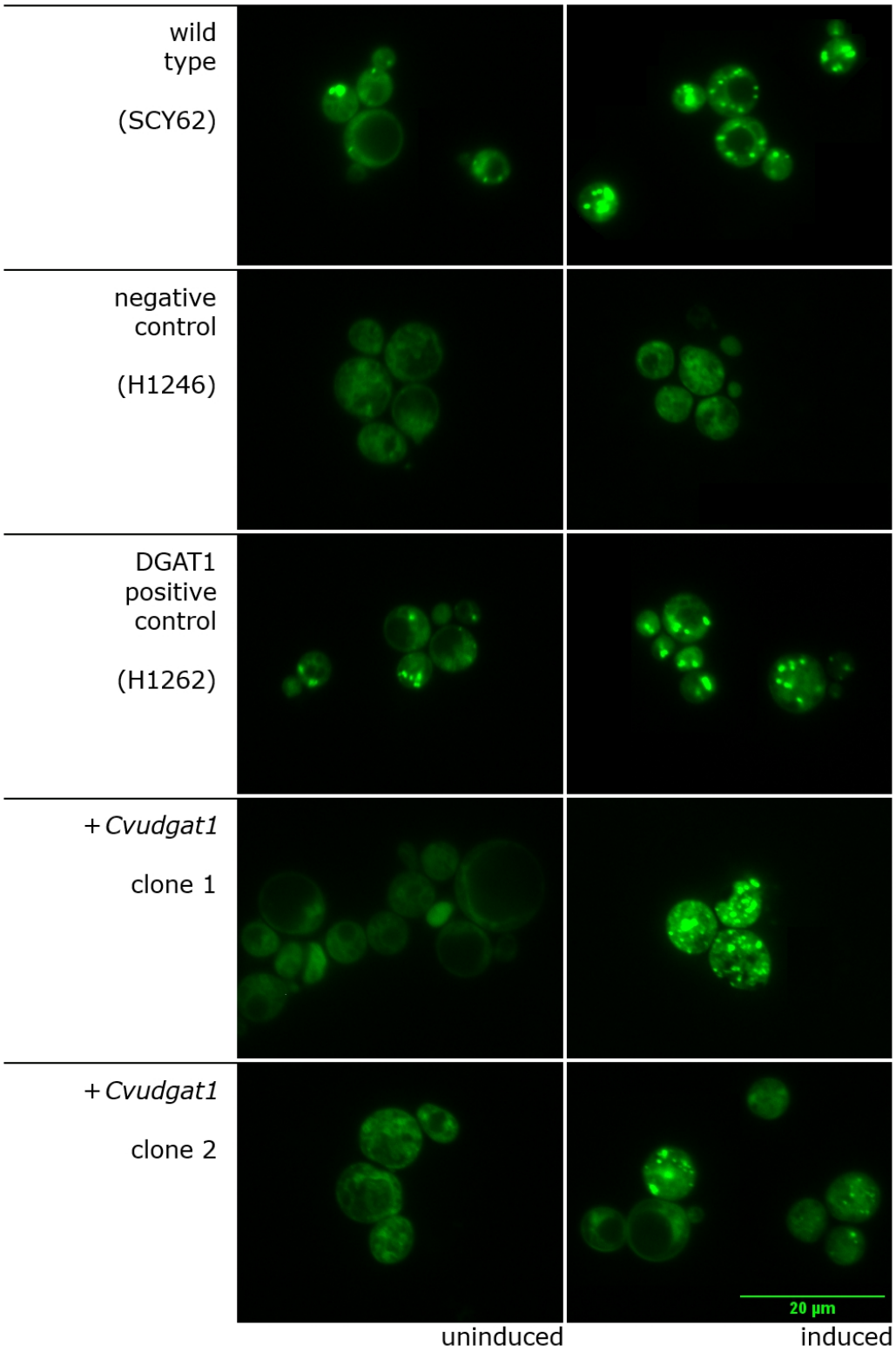


Figure 4.6 | Formation of lipid bodies by *Cvudgat1* expression in a non-oil-producing yeast mutant. Uninduced yeast cells (left panel) and yeast cells induced by galactose (right panel). Yeast strains are described in Section 4.2.4. Table 4.2. Clones 1 and 2 are the quadruple disrupted mutants transformed with the *Cvudgat1* gene (pYES2_*Cvudgat1* construct).

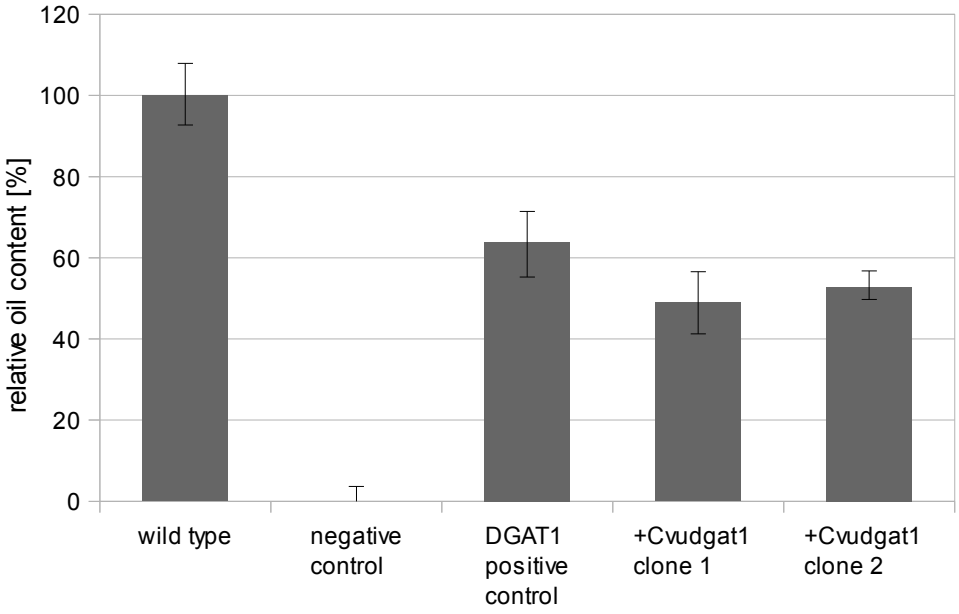


Figure 4.7 | Impact of *Cvudgat1* expression on oil-content of non-oil-producing yeast mutants. All yeast strains grew until the late stationary phase in the presence of galactose, the inducer for the heterologous expression of *Cvudgat1*. The relative oil content was evaluated by flow cytometry, where the non-polar lipids were stained and visualized using Bodipy® 493/503. The relative oil-content of the DGAT1 positive control line and *Cvudgat1* expressing clones (quadruple disrupted mutant +*Cvudgat1* clone 1 and 2) have been normalized with respect to the wild type control (set to 100 %) and the negative control line (quadruple disrupted mutant; set to 0 %; calculation see Chapter 3, Section 3.2.7.). The data combines four independent experiments with three biological replicates. The error bars indicate the fluctuations of the values between 12 replicates.

Table 4.4 | Comparison of relative fatty acid composition in the total lipids of the yeast *S. cerevisiae* and the microalga *C. vulgaris*.

Fatty acid methyl ester (FAME)		*S. cerevisiae*	*C. vulgaris*
palmitic acid	C16:0	15.4 ± 0.1	20.60
palmitoleic acid	C16:1 Δ^{7}	29.8 ± 0.3	1.10
hexacadienoic acid	C16:2 $\Delta^{7, 10}$	n.d.	3.34
hexadecatrienoic acid	C16:3 $\Delta^{7, 10, 13}$	n.d.	4.20
stearic acid	C18:0	9.9 ± 0.2	1.85
oleic acid	C18:1 Δ^{9}	44.6 ± 0.3	38.62
linoleic acid	C18:2 $\Delta^{9, 12}$	0.1 ± 0.1	10.54
α-linoleic acid	C18:3 $\Delta^{9, 12, 15}$	n.d.	18.20
others		n.d.	1.48

The values are listed in percent of total lipid extracted.

4.4. Conclusion

In this study, we identified and characterized the *dgat1* gene from *C. vulgaris* (*Cvudgat1*). CvuDGAT1 is a member of the MBOAT superfamily and shares distinctive motifs of other DGAT1s from microalgae and plants. We identified the binding domains for both substrates, acyl-CoA and DAG, the putative active site, and a phenylalanine (Phe^{430}) found to be essential for DGAT1 enzyme activity. Furthermore, we determined its localization and integration into the ER membrane. We report for the first time the putative tertiary structure of DGAT1. This model clearly shows that DGAT1 is integrated into the ER membrane and that domains required to catalyze the reaction are adjacent, although they are widely separated from each other in the protein sequence. Through functional characterization for *Cvudgat1* in non-oil-producing yeast mutants, we verified that the isolated gene from *C. vulgaris* has DGAT enzyme activity in yeast and restored oil-production to nearly the same levels as the wild type.

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5. Genetic Engineering of Microalgae

In the last two decades, microalgae has been discovered as high-productive cell-factories. Thereby, genetic engineering can even increases its application area and open doors for the production of complex foreign proteins, such as pharmaceuticals or hormones. Furthermore, their metabolism can be altered to increase the production of naturally occurring compounds, such as biodiesel.

For an easy, rapid and efficient overexpression of desired genes, we present here a complete genetic engineering protocol tailored for the green alga *Chlorella vulgaris*. Therefore, we constructed a high efficient expression cassette and tested several transformation methods. We chose the pMDC162 vector and tested in total four promoters for evaluation in *Nicotiana benthamiana* and *Chlamydomonas reinhardtii* via ß-glucuronidase (GUS). The promoters includes the mannopine synthase promoter (MAS), the polyubiquitin-1 promoter (Ubi1), the chimeric heat shock 70A – RuBisCO promoter system (Hsp70A/RBCS2) and the RBCS2 promoter alone. In both species, the MAS and the Hsp70A/RBCS2 promoter showed the strongest expression. To establish an efficient protocol to stably transform *C. vulgaris*, several transformation methods were tested, including electroporation, *Agrobacterium*-mediated gene transfer, and the PEG method.

In a final step, our *gene of interest* (*goi*), diacylglycerol acyltransferase type-1 (DGAT1; Chapter 4), were inserted into the compiled final expression cassette, containing a highly-efficient 5´- and 3´-untranslated region (UTR), within the pMDC162 vector. Ready to test the genetic engineering protocol and analyze the impact of the recombinant gene on the *C. vulgaris* oil-metabolism.

5.1. Introduction

Genetic engineering of microalgae and its scalable cultivation in bioreactors will favor its applicability as cell-factories for producing new bioactive products (i. e. high-quality hormones and antibodies) or enhancing the productivity of naturally occurring compounds (León-Bañares 2004; Zaslavskaia *et al.* 2001).

Unfortunately, it is still challenging to genetically engineer microalgae. The main problem is the delivery and the stable integration of foreign DNA into the algal genome (León and Fernández 2007; León-Bañares 2004). Successful transformation seems to be species-dependent, requiring optimized protocols for each microalgae species due in part to the outer cell wall characteristics of the microalgae species (Radakovits *et al.* 2010). A further problem for foreign gene expression is the biased codon usage of microalgae with a high GC-content (up to 71 %; Jarvis *et al.* 1992; Mayfield and Kindle 1990; Kessler 1976), which restricts its expression efficiency. This fact makes prior codon usage adaptation for many microalgae species inevitable (León and Fernández 2007; León-Bañares 2004; Zaslavskaia, *et al.* 2000; Mayfield and Kindle 1990). The high GC-content could might also result in a deviation of the transcriptional and translational regulatory mechanisms found in other organisms, which could complicate the efficient expression of foreign genes (León and Fernández 2007; León-Bañares 2004; Mayfield and Kindle 1990). These characteristics of microalgae make it difficult to apply microalgae as cell-factories for foreign gene expression.

To circumvent these limitations, homologous genes are applied, which enable efficient expression (Kindle 1990; Mayfield and Kindle 1990). Increasingly, working groups focus on the overexpression of naturally occurring genes, which are assumed to be pathway bottlenecks, to increase the productivity of naturally occurring algae metabolites, such as carotenoids, polyunsaturated fatty acids, hydrogen or triacylglycerols (TAGs; Trentacoste *et al.* 2013; La Russa *et al.* 2012; Moellering and Benning 2010; Del Campo *et al.* 2007; Steinbrenner and Sandmann 2006; Dunahay *et al.* 1996).
Our research is focused on developing a comprehensive engineering protocol to enable the investigation of biosynthetic enzymes involved in oil-biosynthesis of *C. vulgaris*.

The green algae *C. vulgaris* is a unicellular organism of 5 - 10 µm in diameter. Its tough and rigid cell wall enables its stability against environmental influences (Kessler 1976). *C. vulgaris* is fast-growing and can achieve a biomass productivity of up to 200 mg FCW/L/d (fresh cell weight; Mata *et al.* 2010). Its oil-content can be increased up to 40 - 60 % dry cell weight (DCW) by altering abiotic factors (temperature, pH), biotic factors (i. e. nutrient limitation), or the carbon source (phototrophic to mixo- or heterotrophic conditions; Al-Iwayzy *et al.* 2014; Praveenkumar *et al.* 2012; Mallick *et al.* 2012; Mujtaba *et al.* 2012; Kurt 2011; Yeh and Chang 2011; Guarnieri *et al.* 2011; Mata *et al.* 2010; Converti *et al.* 2009; Liu *et al.* 2008; Illman *et al.* 2000). Hence, understanding the regulation of lipid-biosynthesis will enable the engineering of increased oil-productivities in *C. vulgaris*.

To genetically engineer microalgae, a highly-efficient expression cassette within a suitable vector system is required. The vectors commonly used for genetic engineering of microalgae have a size between 9.5 kbp and 16.1 kbp (La Russa *et al.* 2012; Huang *et al.* 2006; Kumar *et al.* 2004; Hawkins and Nakamura 1999; Lumbreras *et al.* 1998; Chen *et al.* 2001; Chow and Tung 1999). The vector should contain a suitable selection system within the T_i -region for selection of positive transformants, as some microalgae species showed insensitivity against certain antibiotics (Hawkins and Nakamura 1999; Chow and Tung 1999).
One of the most crucial steps for efficient gene expression in engineered microalgae is the choice of the promoter, which drives the *gene of interest* (*goi*) expression (Vila *et al.* 2012; León and Fernández 2007; Schroda *et al.* 2000). In *C. reinhardtii*, most endogenous and foreign promoters resulted in poor expression levels (Neupert *et al.* 2012). Currently, the most common and well-studied promoter fusion for *C. reinhardtii* is the Hsp70A/RBCS2 chimeric promoter system (Schroda *et al.* 2000; Strenkert *et al.* 2013; Eichler-Stahlberg *et al.* 2009; Li and Tsai 2009; Chen *et al.* 2008). This system is a fusion of the heat shock protein 70A (Hsp70A) promoter with a downstream attached promoter from the small subunit 2 of ribulose-1,5-bisphosphate carboxylase / oxygenase (RuBisCO; Schroda *et al.* 2000). But also the PsaD promoter (derived from the photosystem I subunit; La Russa *et al.* 2012; Croft *et al.* 2007) and the Ubi1 promoter (derived from polyubiquitin-1; Chen *et al.* 2001; Taylor *et al.* 1993) were found to drive good expression in microalgae. The CaMV 35S was used for the first attempts of engineering microalgae but lost its relevance due to its varying success and lower expression level compared to other developed promoters or promoter systems (Huang *et al.* 2006; Kumar *et al.* 2004;

Kim *et al.* 2002; Chen *et al.* 2001; Hawkins and Nakamura 1999; Chow and Tung 1999; Jarvis and Brown 1991).

The most challenging step for engineering microalgae is the transformation and, thereby, the production of stable transformants. For every microalgae species, the transformation has to be optimized, as the transformation efficiency is strongly species-dependent (Radakovits *et al.* 2010; Hallmann 2007). The efficiency and the number of stable transformants are believed to be inversely correlated with size and the complexity of the alga (Hallmann 2007). Only a few species are routinely transformed, these include *C. reinhardtii*, *Volvox carteri*, the diatom *Phaeodactylum tricornutum,* and several *Chlorella* species (Walker *et al.* 2005; León-Bañares 2004). Recently, an additional transformation protocol for the nuclear DNA-insertion in *Nannochloropsis* has been published (Radakovits *et al.* 2012).
A variety of methods have been applied to transform microalgae. These include the glass-bead method (Kindle 1990), which is similar to the PEG method (Jarvis and Brown 1991), the silicon carbide (SiC) method (Dunahay 1993), electroporation (Maruyama *et al.* 1994), particle bombardment (Mayfield and Kindle 1990), and the *Agrobacterium*-mediated transformation method (Kumar *et al.* 2004). For *Chlorella* species, the most frequent applied transformation methods are electroporation (Maruyama *et al.* 1994; Huang *et al.* 2006; Chen *et al.* 2001; Chow and Tung 1999) and the PEG-method (Kim *et al.* 2002; Huang *et al.* 2006; Hawkins and Nakamura 1999). Just recently, a publication about an *Agrobacterium*-mediated transformation was published for *C. vulgaris* (Cha *et al.* 2012).

In this study, we established an efficient expression system by evaluation of four different promoters, including the mannopine synthase promoter (MAS), the polyubiquitin-1 promoter (Ubi1), the chimeric heat shock 70A – RuBisCO promoter system (Hsp70A/RBCS2) and the RBCS2 promoter. Then we inserted them into a high-efficient 5´- and 3´- end expression cassette within the pMDC612 vector to test in a next step several transformation methods to achieve a complete and efficient genetic engineering protocol for *C. vulgaris* allowing us to overexpress the desired *goi* easily and rapidly.

5.2. Material and Methods

5.2.1. Microalgae Strains

In this study, we used *Chlorella vulgaris* (UTEX-259 and UTEX-395) and *Chlamydomonas reinhardtii* (UTEX-2244) strains. The C. *vulgaris* UTEX-259 strain was obtained from *UTEX - The Culture Collection of Algae* (Austin, USA) and cultured as described in Chapter 3, Section 3.2.1.1. The strains C. *reinhardtii* and C. *vulgaris* UTEX-395 were kindly provided by Dr. Stefanie Grade (Institute of Biological Production Systems, Leibniz University Hanover (LUH); Germany). *C. reinhardtii* were cultured in ES-media[1] and *C. vulgaris* were cultured in fertilizer (Chapter 3, Section 3.1.4A). Both cultures were cultivated at 24 ± 2 °C by agitating at 180 rpm at a photon flux density of 50 µmol/m^2/s provided by cool white fluorescent bulbs.

5.2.2. Plasmid Construction for Promoter Evaluation

The promoters were obtained from the following plasmids: (1) MAS promoter from the pGreenII0229TR_MAS_*gus-luc* (kindly provided by Dr. Heiko Kiesecker, *Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures*), (2) Hsp70A/RBCS2 promoter from the pCB740 (*Chlamydomonas Resource Center*, University of Minnesota, USA), and (3) Ubi1 promoter (kindly provided by Sean Flanigan, Interim Director of *Technology Transfer & Business Enterprise* of the University of Ottawa, Canada).

We first transferred the MAS_*gus* construct from the pGreenII0229TR_MAS_*gus_luc* (8,807 bp) to the smaller pUC18 cloning vector (2,686 bp) by amplifying MAS_*gus* with the primers_15 and _16 (Chapter 3; Section 3.1.8., Table 3.7). The 5´-ends of the forward and reverse primer contain a SacI and HindIII restriction site, respectively. Both are present in the multiple cloning site of pUC18. After the successful insertion of MAS_*gus* in pUC18, the MAS promoter was exchanged with the Ubi1, Hsp70A/RBCS2, and RBCS2 promoters. For the RBCS2 promoter, the exchange was performed with the restriction enzymes ClaI and XbaI, by PCR amplification of RBCS2 with primers containing the corresponding restriction enzymes sites in their 5´-end sequences (primer_14/13; Chapter 3; Section 3.1.8., Table 3.7). The Ubi1 and the Hsp70A/RBCS2 promoter exchange were performed with an overlap extension PCR (OE-PCR; Chapter 3, Section 3.2.4.5.; Ubi1: primer_28/29; Hsp70A/RBCS2: primer_30/31; Chapter 3; Section 3.1.8., Table 3.7), as both promoters showed an internal XbaI restriction site (Ubi1: position 946; Hsp70A/RBCS2: position 58). The right insertion of all promoters was verified by sequencing.

Next, we transferred the four *promoter_gus* constructs from the pUC18 plasmid into pMDC162 with removed Gateway system and *gusA* gene (pMDC162_w/oGWsys; Chapter 3; Section 3.1.9.). Within the T_i-region, between the nos terminator and the right border are the restriction sites for SacI (cuts after base 275), KpnI (cuts after base 281), and PstI (cuts after base 308). Those sites were used to insert the four *promoter_gus* constructs. First, all four constructs were amplified by PCR using primers that contain restriction enzymes sites for a reverse directed insertion in pMDC162_w/oGWsys, so that

1 recipe: http://sagdb.uni-goettingen.de/culture_media/01%20Basal%20Medium.pdf; status Sept. 29th 2014

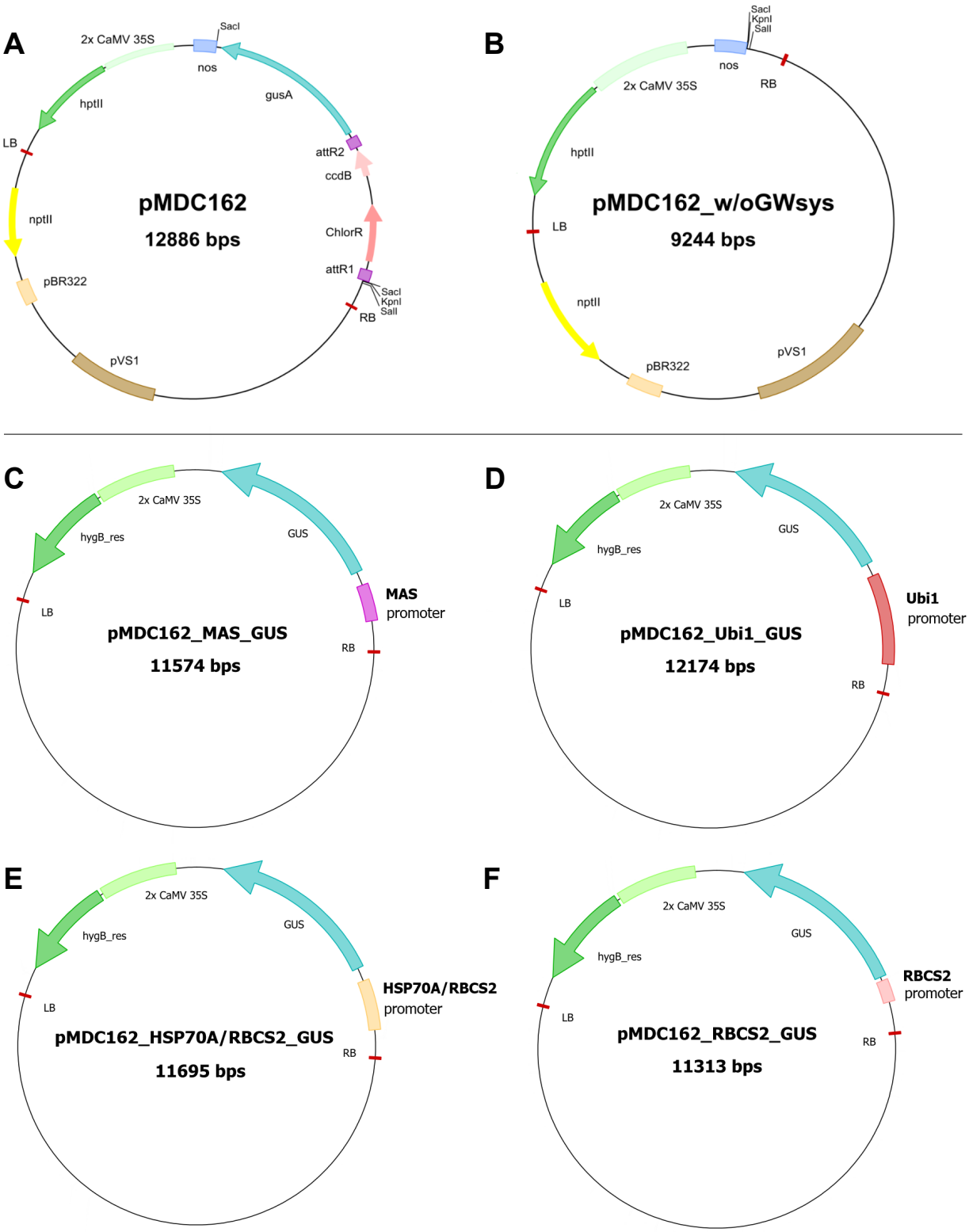

Figure 5.1 | pMDC162 vectors used for promoter evaluation. A, original pMDC162 vector (Curtis and Grossniklaus 2003); **B**, pMDC162 with removed Gateway system and *gusA* gene by cutting the plasmid twice with SacI and re-ligation of the plasmid. **C - F**, plasmids containing the four *promoter_gus* constructs for promoter evaluation (**C**, MAS; **D,** Ubi1; **E**, Hsp70A/RBCS2; **F**, RBCS2). *pBR322*: replicon for *E. coli*; *pVS1*: replicon for *A. tumefaciens*; *nptII*: kanamycin resistance gene encoding neomycin phosphotransferase; *hptII*: hygB resistance gene encoding hygromycin phosphotransferase; *nos*: nos terminator; *gusA*: ß-glucuronidase gene; *attR1* and *attR2*: recombination sites 1 and 2; *ccdB*: toxin gene for type II toxin-antitoxin system; *ChlorR*: chloramphenicol resistance; *RB*: right border; *LB*: left border.

the nos is able to terminate *gus* expression. In all primers sets, except those for Hsp70A/RBCS2_*gus* amplification, the forward and reverse primer contains an attached KpnI and SacI restriction site within their 5´-ends, respectively (MAS_*gus*: primer_47/45; Ubi1_*gus*: primer_48/45; RBCS2_*gus*: primer_50/45; Chapter 3; Section 3.1.8., Table 3.7). For amplification and cloning of the Hsp70A/RBCS2_*gus* construct, the forward primer contained an attached PstI and the reverse primer a KpnI restriction site (primer_49/46; Chapter 3; Section 3.1.8., Table 3.7). After digestion of the four amplified *promoter_gus* constructs and the vector pMDC162_w/oGWsys with the corresponding restriction enzymes, the final vectors were assembled in a ligation reaction and verified by sequencing. The produced constructs are pMDC162_w/oGWsys_MAS_*gus*, pMDC162_w/oGWsys_Ubi1_*gus*, pMDC162_w/oGWsys_ Hsp70A/RBCS2_*gus*, and pMDC162_w/oGWsys_RBCS2_*gus* (Figure 5.1) and were used for promoter evaluation in *N. benthamina* and *C. reinhardtii* (Section 5.3.1.2.).

5.2.3. Transformation Protocols for Promoter Evaluation

N. benthamiana seedlings were transiently transformed by applying the Fast *Agrobacterium*-mediated Seedling Transformation protocol (FAST-method) according to (Li *et al.* 2009; Weaver *et al.* 2014). *N. benthamiana* seeds (50 mg) were sterilized in 0.5 % sodium hydrochloride containing 0.1 % Tween 20 for 10 min, followed by 70 % ethanol for 1 min, and finally rinsed with sterile H_2O five times for 30 sec each. Seeds were germinated on Murashige-Skoog- (MS-) solid media plates (Chapter 3, Section 3.1.2., Table 3.1) under 16 hours light / 8 hours dark at 22 °C. For transformation, the *N. benthamiana* seedlings (8 days after planting) were co-cultivated with the *A. tumefaciens* containing our four pMDC162_*promoter_gus* constructs (Section 5.2.2.) for 50 hours in the dark. The seedlings were stored for another 24 hours in light at 22 °C to express the recombinant gene before the GUS-assay was performed (Section 5.2.4.).

The transformation of *C. reinhardtii* requires the production of protoplasts. For protoplast formation, *C. reinhardtii* cultures (50 mL) at a density of 2 x 10^7 cells/mL was harvested by centrifugation at 1,500 xg for 5 min. The cell pellet was washed once with 25 mM sodium phosphate buffer (pH = 6) and then resuspended in 5 mL *cell-wall-digestion* buffer (Chapter 3, Section 3.1.2., Table 3.1). Prior to digestion the enzymes 4 % w/v cellulase, 2 % w/v macerase, and 50 units pectinase were added. The cell suspension was incubated for 16 hours at 25 °C in the dark by gentle shaking (50 rpm). The *C. reinhardtii* protoplasts were transformed using the polyethylene glycol (PEG) method according to Kim *et al.* (2002; described in detail in Section 5.2.5.). After transformation, the *C. reinhardtii* cells were resuspended in 600 µL *regeneration media* (Chapter 3, Section 3.1.2., Table 3.1) and incubated for 23 hours at 25 °C in the dark. For expression of the recombinant gene, *expression-culture*-media (1 mL; Chapter 3, Section 3.1.2., Table 3.1) was added to the cell suspension and incubated for 30 hours at 25 °C and 120 rpm prior to GUS-assay (Section 5.2.4.). Two hours before starting the GUS-assay, the culture containing the pMDC162_w/oGWsys_Hsp70A/RBCS_*gus* construct (Section 5.2.2.) was heat-shocked for 40 min at 40 °C according to the protocol by Schroda *et al.* (2000).

5.2.4. GUS-Assay

N. benthamiana seedlings (10 - 60 seedlings) were transferred into a 50 mL reaction tube and incubated with 90 % acetone (40 mL) for 20 min. After washing several times with H_2O, the seedlings were immersed in fresh staining solution (40 mL; Chapter 3, Section 3.1.2., Table 3.1) and incubated for 16 hours in the dark at 37 °C. Then, the seedlings were incubated in 70 % v/v ethanol (40 mL) for another 1 - 3 days.

C. reinhardtii cells were harvested by centrifugation at 3,000 xg for 3 min and incubated for 20 min in pre-chilled acetone. Cells were washed twice with 500 µL H_2O, resuspended in 500 µL fresh staining solution (Chapter 3, Section 3.1.2., Table 3.1), and incubated for 16 hours in the dark. Afterwards, the cells were centrifuged for 3 min at 5,000 xg and resuspended in 500 µL 70 % v/v ethanol until evaluation.

5.2.5. Transformation of *C. vulgaris*

Electroporation

C. vulgaris cultures (20 mL) at a density of 2 x 10^7 cells/mL were harvested by centrifugation (450 xg, 3 min). The cell pellet was washed once with BBM (Chapter 3; Section 3.1.4.A) and resuspended in 2 mL H_2O. Then, 80 µL of cell suspension was transferred with 5 µg plasmid DNA in a pre-chilled electroporation cuvette with a 0.1 cm gap between the two electrodes and incubated for 5 min on ice. The electric shock were performed in a MicroPulser™ Electroporator (BioRad) with a capacitance at 25 µF, a resistance of 200 Ω, and an electric pulse of 1500 V/cm, 1800 V/cm or 2000 V/cm. After the transformation procedure, the cells were stored on ice for 2 min, transferred to 15 mL reaction tubes with 2 mL BBM, incubated for two hours at 24 °C and 250 rpm in the dark, and spread on BBM-plates (Chapter 3; Section 3.1.4.A).

Agrobacterium-mediated transformation

The *Agrobacterium* culture were freshly grown in YEP-media up to a density of OD_{600} 0.8 - 1.2. Then, 800 µL were harvested by centrifugation (12,000 xg, 30 s), washed twice with 500 µl and 100 µL *induction* media (Chapter 3, Section 3.1.2., Table 3.1) and added to the microalgae lawn. The microalgae lawn was obtained by first grow *C. vulgaris* in BBM for 4 days, harvest 1.5 mL (equals about 10^7 cells; 3,000 xg, 3 min), resuspend algal pellet in 100 µL BBM and plate them on co-cultivation media plates (Chapter 3, Section 3.1.2., Table 3.1). Here, they grew for 2 days on light (16:8 photo period) before 100 µL of the *Agrobacterium*-suspension were added and spread with glass-beads. The approaches were co-cultivated at 25 °C for 3 days in the dark. To remove the *Agrobacterium* the cells were resuspended in 1 mL BBM supplemented with timentin (100 µg/mL). The algal-*Agrobacterium* suspension were removed with the pipette and transferred into a new tube. This step was repeated until about 3 mL were collected. The suspension were incubated in a shaking incubator (200 rpm) with no direct illumination for two days. For selection of the transgenic cells, the approaches were centrifuged at 10,000 xg for 1 min. Then, the pellet were resuspended in 400 µL BBM and plated on two BBM-plates supplemented with 5 µg/mL hygromycin B. Remaining cells were directly used for GUS-assay (Section 5.2.4.).

PEG-method

For the formation of protoplasts the same protocol than for *C. reinhardtii* were used. The cell density prior to harvest was about 4 x 10^8 cells/mL. For transformation, we followed the protocol according to Kim *et al.* (2002). Therefore, the protoplast were harvested by centrifugation at 400 xg for 5 min and washed once with Schenk & Hildebrandt media (SH-) media containing 0.3 M D-sorbitol, 0.3 M D-mannitol, and 0.25 % w/v glucose (Chapter 3, Section 3.1.2., Table 3.1). Then, the cells were taken up in 1 mL *protoplast*-solution (Chapter 3, Section 3.1.2., Table 3.1). 140 µL of the solution containing the protoplasts were transferred into a new 1.5 mL tube and 5 µg plasmid DNA together with 25 µg calf-thymus DNA were added. The approaches were incubated for 15 min at room temperature. Then, 200 µL of pre-chilled *PNC*-solution (Chapter 3, Section 3.1.2., Table 3.1) were added, before incubating the approaches for 30 min on a rocker. Finally, 600 µL *regeneration*-media (Chapter 3, Section 3.1.2., Table 3.1; osmolarity: 864 mmol/kg) and incubated for 23 hours at 25 °C in the dark. On the next day, the approaches were transferred into a 50 mL reaction tube with 15 mL of *expression-culture*-media (Chapter 3, Section 3.1.2., Table 3.1; osmolarity: 771 mmol/kg) and incubated at 25 °C and 120 rpm for expression of the recombinant genes.

5.2.6. Setup of the Final Expression Cassettes

For setting up the final expression cassette we used an optimized 5´- and 3´-untranslated region (UTR) and with tags fused to the 3´-end of the gene for detection and purification of the expressed protein. Dr. Thomas Reinard, manager of the biochemistry working group in the Department of Plant Biotechnology at Leibniz University Hanover (LUH; Germany), often use a strong expression cassette for recombinant expression of interleukins in tobacco (not published). Its 5´-UTR contains a poly(CAA), an omega-octamer, and a CT-motif according to de Amicis *et al.* (2007). The 3´-UTR contains the terminator of the small subunit of RuBisCO from soybean (*Glycine max*). A *Chrysanthemum* expression cassette including the RuBisCO terminator showed to be a stronger than those containing a CaMV 35S terminator (Outchkourov *et al.* 2003).

Both UTRs are present in the binary vector system pCleanIPG_2x35S-*smGFP*_ER (Chapter 3, Section 3.1.9., Table 3.8). Additionally, a flag-tag, stepII-tag, and a 6x his-tag are present downstream to the insertion site, followed by an *amber* stop codon (UAG) and the RuBisCO 3´-UTR (Figure 5.2.). The tags will be used for identification and purification of the synthesized recombinant protein.

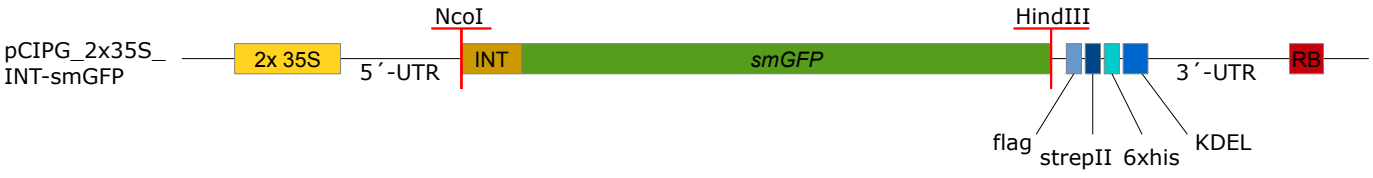

Figure 5.2 | Vector map of the right part of the T_i-region of pCIPG_2x35S_INT-*smGFP*. Shown is the section of the T_i-region which contains the expression cassette we want to use in this study.

Construction of Four High-Powered Expression Cassettes

In a first step the INT-*smGFP* site (1,057 bp) of the pCIPG_2x35S_INT-*smGFP* were removed with NcoI and HindIII and replaced by a self-designed linker (23 bp, Figure 5.3). The linker contains a further restriction site for Kpn2I. This additional restriction site was required, as NcoI is the only restriction site available between the 5´-UTR and the INT-*smGFP* construct (Figure 5.2) and, unfortunately, our *goi*, diacylglycerol acyltransferase type-1 from *C. vulgaris* (*Cvudgat1*), contains an internal NcoI site at position 476, making the NcoI enzyme useless for the final integration. After the insertion of the linker we obtained the plasmid pCIPG_2x35S_linker (Figure 5.3).

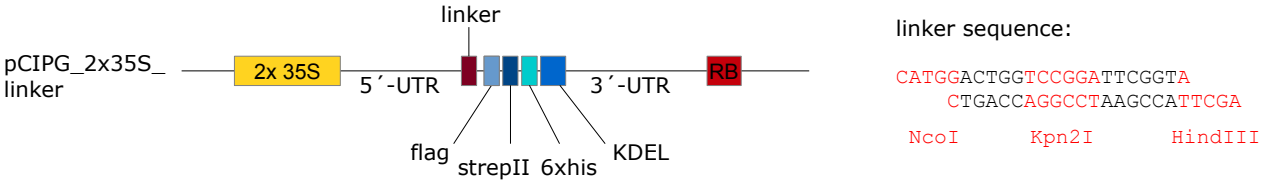

Figure 5.3 | Vector map of the right part of the T_i-region of pCIPG_2x35S_linker. (**A**) Shown is the section of the T_i-region which contains the high efficient expression cassette. The INT-*smGFP* gene were exchanged for a linker containing the restriction sites NcoI, Kpn2I and HindIII. (**B**) Shown is the linker sequence containing the additional restriction site for Kpn2I.

After verification of the pCIPG_2x35S_linker sequence by sequencing, we replaced the 2x 35S promoter with the four promoters MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 (Section 5.2.2.). The promoters were amplified using the appropriate primers (Chapter 3; Section 3.1.8., Table 3.7). The 5´-ends of each forward and reverse primer contains a KpnI and EcoRI restriction site, respectively.

The digested and purified promoters were each inserted in a ligation reaction with the digested vector pCIPG_2x35S_linker containing the wanted expression cassette. In this cloning step we obtained the four plasmids pCIPG_MAS_linker, pCIPG_Ubi1_linker, pCIPG_Hsp70A/RBCS2_linker, and pCIPG_RBCS2_linker (Figure 5.4). The correct insertion was verified by sequencing.

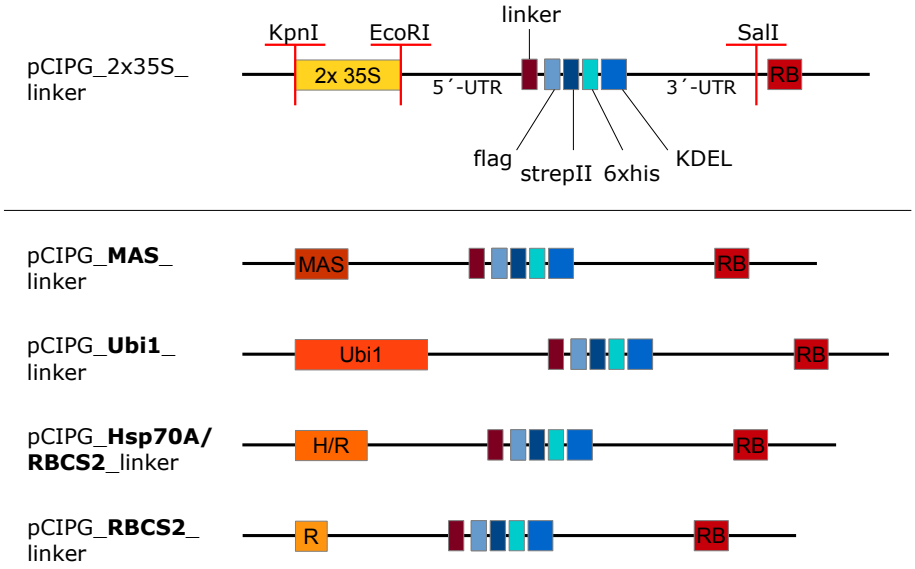

Figure 5.4 | Promoter exchange within the plasmid pCIPG_2x35S_linker. The 2x35S promoter were removed with the restriction enzymes KpnI and EcoRI and exchanged with the promoters MAS, Ubi1, Hsp70A/RBCS2 (H/R) and RBCS2 (R) in four approaches.

This cloning step completed the section of the preparation of high powered expression cassettes containing the promoters MAS, Ubi1, Hsp70A/RBCS2, and RBCS2. For transferring the entire expression cassette to another vector, it can easily be removed with the enzymes KpnI and SalI, cutting directly upstream and downstream of the promoter and terminator region, respectively. The sizes of the complete expression cassettes without *goi* are 881 bp for the MAS promoter, 1,481 bp for the Ubi1 promoter, 1,023 bp for the Hsp70A/RBCS2 promoter, and 723bp for RBCS2 promoter. Originating from these four constructs, every *goi* can be inserted in a single cloning step with the restriction enzymes combinations NcoI/Kpn2I, NcoI/HindIII, or Kpn2I/HindIII.

5.2.6.1. Construction of Final Vectors Driving the Gene of Interest

To construct the final pMDC162_w/oGWsys vectors containing the four previously constructed expression cassettes (Section 5.3.1.5.) and our *goi*, the *Cvudgat1* gene, we have to follow two different strategies. The *Cvudgat1* gene contains a NcoI restriction site at position 476, which requires the insertion in the linker region with the enzymes Kpn2I/HindIII. Unfortunately, the RBCS2 promoter contains the Kpn2I restriction site at position 100. In this way also the Hsp70A/RBCS2 will be cut at position 400 when cloning with Kpn2I. Caused by this, we have to pursue a different strategy for the expression cassettes containing the Hsp70A/RBCS2 or the RBCS2 promoter, in contrast to those containing the MAS and Ubi1 promoter.

For the expression cassettes inhibiting the MAS and Ubi1 promoter, the *Cvudgat1* gene were amplified by PCR (primer_59 + 60; Chapter 3; Section 3.1.8., Table 3.7), in which the primers contain the Kpn2I and HindIII enzyme sites in their flanking regions, respectively. Those sites were used to insert the *Cvudgat1* construct into the linker region. Resulting in the constructs pCIPG_MAS_*Cvudgat1* and pCIPG_Ubi1_*Cvudgat1*, which were verified by sequencing. Then, the expression cassettes of both vectors were transferred to pMDC162_w/oGWsys with KpnI and SalI. The final constructs pMDC162_w/oGWsys_MAS_*Cvudgat1* (from now on called pMDC162_MAS_ *Cvudgat1*) and pMDC162_w/oGWsys_Ubi1_*Cvudgat1* (from now on called pMDC162_Ubi1_ *Cvudgat1*) were confirmed by sequencing. A simplified overview about the constructed T_i-regions are shown in Figure 5.5.

To obtain the final vector containing the Hsp70A/RBCS2 and the RBCS2 promoter driving the *Cvudgat1* gene, a reverse cloning strategy were performed. We first inserted the *Cvudgat1* gene in the plasmid pCIPG_2x35S_linker with Kpn2I and HindIII. Then, the 2x 35S promoter were exchanged for the Hsp70A/RBCS2 and RBCS2 promoter by using KpnI and EcoRI. In a final step, both expression cassettes were transferred into pMDC162_w/oGWsys with the restriction enzymes KpnI and SalI. All cloning steps were verified by sequencing. The T_i-regions of the vectors pMDC162_w/oGWsys_ Hsp70A/RBCS2_*Cvudgat1* (from now on called pMDC162_Hsp70A/RBCS2_*Cvudgat1*) and pMDC162_w/oGWsys_RBCS2_*Cvudgat1* (from now on called pMDC162_RBCS2_*Cvudgat1*) are shown in Figure 5.5.

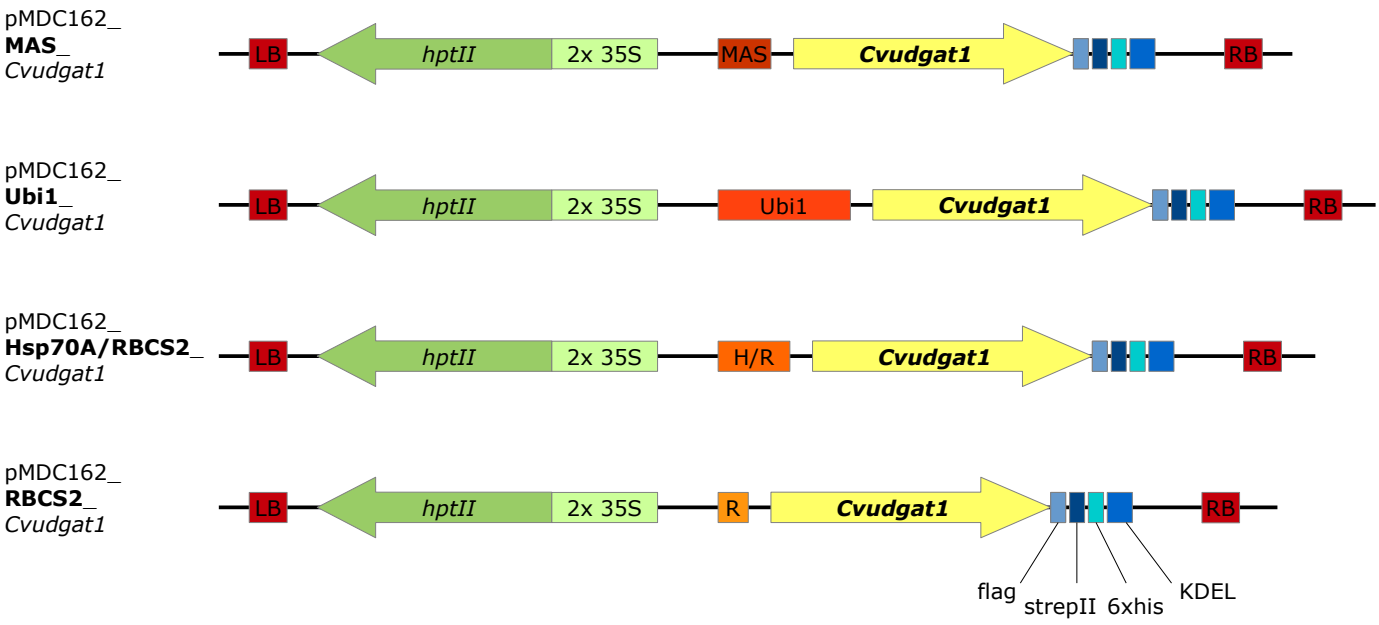

Figure 5.5 | Overview of the T_i-regions within the four expression cassettes driving the *Cvudgat1* gene pMDC162_w/oGWsys. To obtain the constructs two different cloning strategies were performed. For the expression cassettes containing the promoters MAS and Ubi1 the *Cvudgat1* gene were inserted in the expression cassette. In a next step, the entire cassette were inserted in pMDC162_w/oGWsys (see text for details). To obtain the Hsp70A/RBCS2 (**H/R**) and RBCS2 (**R**) driving the *Cvudgat1* gene in pMDC162 a reverse cloning strategy had to be pursued. The *Cvudgat1* gene were first inserted to pCIPG_2x35S_linker, then the promoter were exchanged, and the expression cassette transferred to pMDC162_w/oGWsys (see text for details).

The sizes of the final four pMDC162 vectors are 11,482 bp, 12,082 bp, 11,624 bp, and 11,324 bp containing the MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 promoter, respectively (Chapter3, Section 3.1.9., Table 3.8). The appropriate T_i-regions showed sizes of 5,249 bp, 5,849 bp, 5,391 bp, and 5,091 bp.

5.3. Results and Discussion

5.3.1. Construction of a High-Powered Expression Cassette

In this section we constructed an expression cassette for efficient recombinant gene expression in *Chlorella vulgaris*.

A suitable selection system should already be present in the chosen vector. For a high expression level of the recombinant gene, promoters were further investigated by evaluation with the reporter ß-glucuronidase gene (*gus*) reporter gene in the dicotyledon *Nicotiana benthamiana* and the green algae *Chlamydomonas reinhardtii*. Subsequently, a strong expression cassette with highly-efficient 5´- and 3 ´-ends were constructed and inserted into the chosen vector. Our ultimate goal was to construct an expression cassette to drive our *gene of interest* (*goi)*, diacylglycerol acyltransferase type-1 (DGAT1; Chapter 4), and demonstrate its impact of engineering on the oil-biosynthetic pathway in engineered *C. vulgaris*.

5.3.1.1. Selection of Selection System

The selection system for *C. vulgaris* should be well-established for microalgae and suitable for *C. vulgaris*, and also effective at low concentrations in Basal Bolds media (BBM; Chapter 3 Section 3.1.4.A). Established selection agents in *Chlorella* species are phleomycin applied for *C. ellipsoidea* (Kim *et al.* 2002), gentamycin (G418) for *C. ellipsoidea* (Chen *et al.* 2001), *C. vulgaris,* and *C. sorokiniana* (Hawkins and Nakamura 1999)), and hygromycin B (hygB) for selection of *C. vulgaris* (Chow and Tung 1999) and *Chlorella* sp. DT (Huang *et al.* 2006).

To find an appropriate selection system for *C. vulgaris* we tested the toxicity level of the antibiotics hygB, kanamycin (kan), and spectomycin (spec) at three concentrations (5, 10, and 25 µg/mL; Figure 5.6).

In the toxicity test, hygB showed no impact on the cell growth at the concentration of 5 µg/mL. Cell growth was restricted at a concentration of 10 µg/mL and completely inhibited at 25 µg/mL hygB (Figure 5.6). The antibiotic kan was not toxic for *C. vulgaris* at all applied concentrations. The cells grew similarly to the positive control. *C. vulgaris* cells also grew normally with 5 or 10 µg/mL spec compared to the positive control with slight growth inhibition at 25 µg/mL (Figure 5.6).

In conclusion, the results indicated that *C. vulgaris* has high resistance for several antibiotics. Only hygB inhibited cell growth completely at a concentration of 25 µg/mL. Spec will need a higher concentration than 25 µg/mL to have a toxic effect on *C. vulgaris*. These findings are similar to Hawkins and Nakamura (1999) and Chow and Tung (1999). They reported an insensitivity of *C. vulgaris* against some common antibiotics used for selection, i. e. ampicillin, chloramphenicol, kanamycin, and streptomycin (Hawkins and Nakamura 1999), (Chow and Tung 1999). We will use hygB as selection agent of transformed *C. vulgaris*. HygB also was described to be an effective antibiotic for selection of *C. vulgaris* (Chow and Tung 1999). Although the literature reported a concentration of 50 µg/mL hygB (Huang *et al.* 2006), (Chow and Tung 1999), we found a concentration of 25 µg/mL was already sufficient.

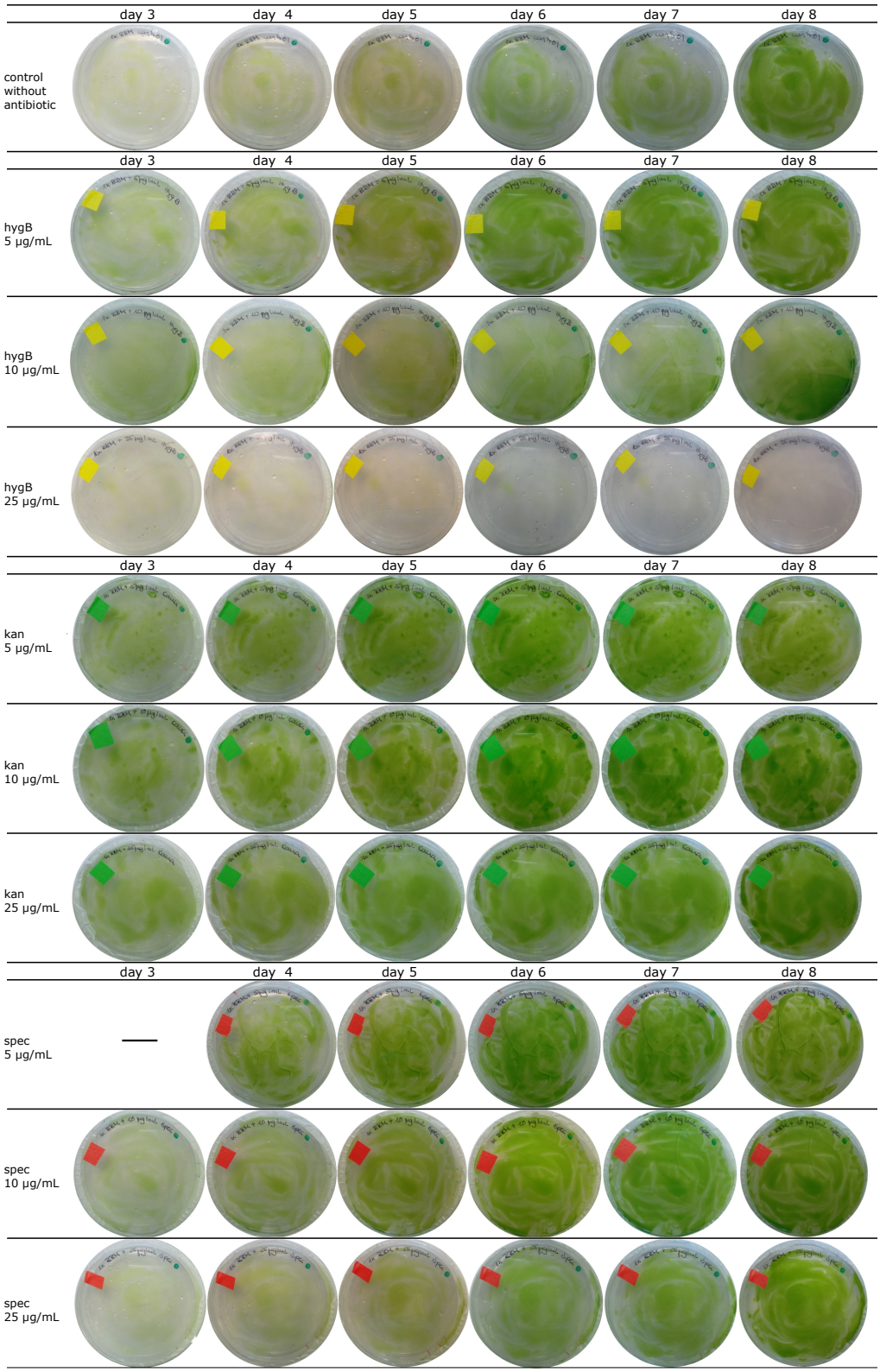

Figure 5.6 | Toxicity test of *C. vulgaris* against three antibiotics at three concentrations. Antibiotics tested include hygromycin B (hygB), kanamycin (kan) and spectomycin (spec). Concentrations of 5, 10 and 25 µg/mL were used. Initially, 8 x 10^7 cells were spread on each plate. Pictures were made every 24 hours starting on day three.

5.3.1.2. Promoter Evaluation

To determine a strong promoter for *C. vulgaris,* we decided to test several promoter systems, including the polyubiquitin-1 promoter (Ubi1) from maize, the chimeric heat shock 70A – RuBisCO promoter system (Hsp70A/RBCS2) from *C. reinhardtii*, the RBCS2 promoter alone, and the mannopine synthase promoter (MAS).

The **Ubi1** promoter is a constitutive promoter but is further strongly induced by heat shock and other stresses (Streatfield *et al.* 2004; Christensen *et al.* 1992). This promoter efficiently enabled *gus* expression in *C. ellipsoidea* (Chen *et al.* 2001), making this promoter an interesting candidate in the *Chlorella* species. The **RBCS2** promoter naturally drives the expression of the small subunit 2 of ribulose-1,5-bisphosphate carboxylase / oxygenase (RuBisCO; Eichler-Stahlberg *et al.* 2009; Lumbreras *et al.* 1998). RBCS2 itself is very weakly inducible by light (Schroda *et al.* 2000). More commonly, RBCS2 is fused downstream of the Hsp70A promoter (**Hsp70A/RBCS2**; Eichler-Stahlberg *et al.* 2009; Schroda *et al.* 2000; Lumbreras *et al.* 1998). The chimeric constellation of Hsp70A/RBCS2 is strongly inducible by heat shock (Schroda *et al.* 2000) and is one of the most frequently used promoter fusions applied for recombinant gene expression in *C. reinhardtii* (Strenkert *et al.* 2013) and other microalgae (Li and Tsai 2009; Chen *et al.* 2008). The constitutive **MAS** promoter has been derived from the TR promoter found on the T_i-plasmid of *A. tumefaciens* (Langridge *et al.* 1989; Velten *et al.* 1984). Originally, the TR-promoter controls the expression of enzymes for the synthesis of the opine mannopine in *A. tumefaciens*-infected plant tissues (Guevara-Garcia *et al.* 1999). Increased induction is caused by wounding (possibly through involvement of ethylene), auxin, and especially cytokinin, which strongly enhances the expression of MAS-regulated genes (Langridge *et al.* 1989). The phytohormone methyl jasmonate has been identified as a suppressor of expression (Guevara-Garcia *et al.* 1999). To our best knowledge, the MAS promoter has not been applied in recombinant gene expression in microalgae.

We evaluated the expression efficiencies of the four promoters, MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 with the reporter gene *gus*, as it is commonly used in microalgae (Chow and Tung 1999; Chen *et al.* 2001; Kumar *et al.* 2004; Huang *et al.* 2006). Therefore, we inserted the promoters and the *gus* gene into the vector pMDC162_w/oGWsys (Section 5.2.2.). For evaluation we used the following methods: (1) the transient *Agrobacterium*-mediated transformation method (FAST-method) in *N. benthamiana* (Section 5.2.3.), and (2) the polyethylene glycol (PEG-) method in the well-studied microalgae *C. reinhardtii* (Section 5.2.3.).

5.3.1.2.1. Promoter Evaluation in *N. benthamiana*

N. benthamiana seedlings were transiently transformed with the pMDC162 vectors containing the four *promoter_gus* constructs (Section 5.2.2.) using the FAST method (Section 5.2.3) followed by the GUS-assay (Section 5.2.4.). The transformation efficiency (A) and the intensity of the GUS stain in each seedling (B) were measured to assess the promoter strength.

(A) Transformation efficiency

In this evaluation method every seedling was assigned as either "*not transformed – no visible GUS staining*" or "*transformed – visible GUS staining*" (Table 5.1; Figure 5.7). Seedlings co-cultivated with and without the wild-type *A. tumefaciens* (unarmed, not containing a binary plasmid) served as the negative controls. As shown in Table 5.1, no endogenous GUS activity in the *N. benthamiana* seedlings or wild-type *A. tumefaciens* was observed in the negative controls. Hence, any GUS staining was attributed to the heterologous expression of *gus*, legitimizing the evaluation of the GUS-assay for promoter assessment in *N. benthamiana.*

Table 5.1 | Transformation efficiency of the FAST-method with *N. benthamiana* seedlings for promoter evaluation.

Promoter	Total number of Seedlings → no GUS stain visible	Total number of transformed seedlings → GUS stain visible	Transformed seedlings [%]
MAS	58	50	86
Ubi1	61	41	67
Hsp70A/RBCS2	61	58	95
RBCS2	59	34	58
neg. control *+A. tumefaciens*	50	0	0
neg. control *-A. tumefaciens*	39	0	0

Table details the total number of seedlings tested for each promoter construct and the negative controls, total number of seedlings showing any GUS staining, and the percentage of transformed seedlings.

Both negative controls, seedlings in co-cultivation with an unarmed or without *A. tumefaciens* in the co-cultivation media, show no level of *gus* expression, so that all detectable GUS stained spots found in the tested seedlings were archived from the heterologous expressed *gus*. This legitimize the evaluation of the GUS test for promoter assessment in *N. benthaminana*, graphically shown in Figure 5.7.

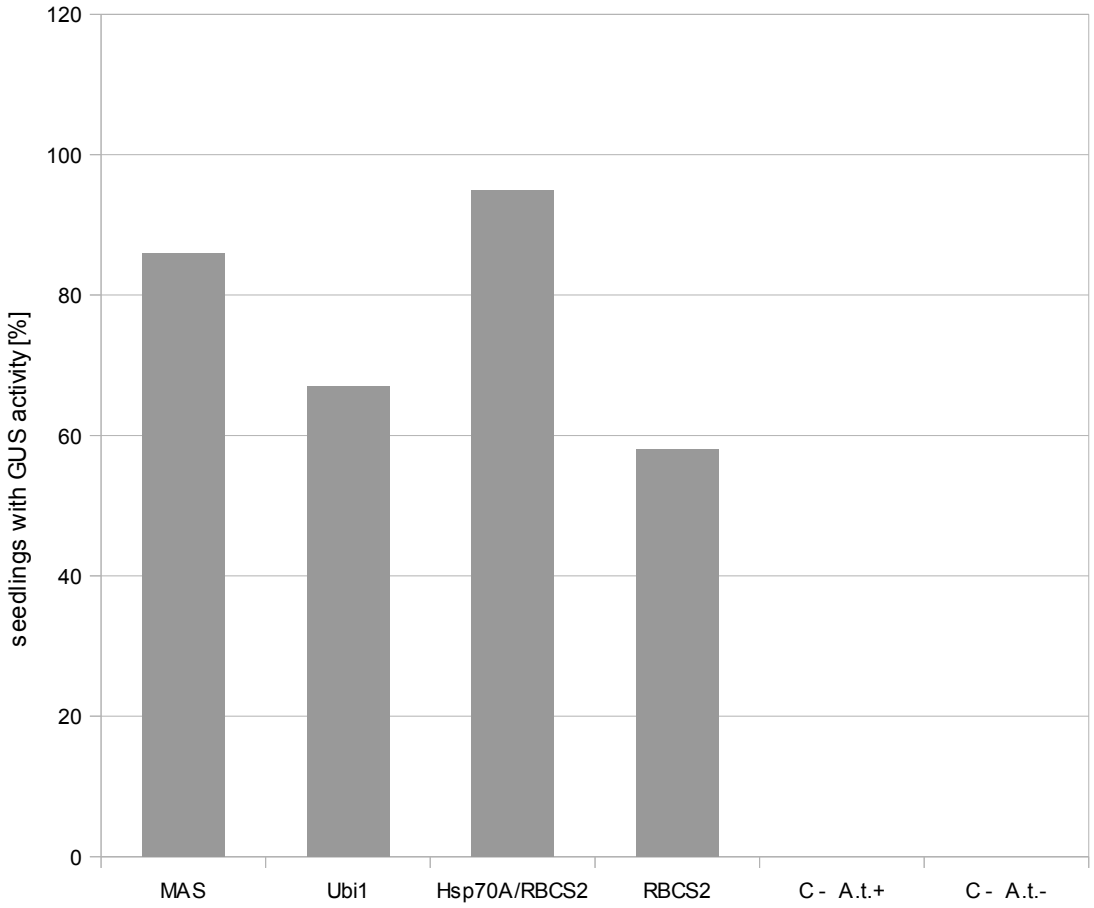

Figure 5.7 | Transformation efficiency of the FAST-method with *N. benthamiana* seedlings for promoter evaluation. The percentage of successfully transformed seedlings with detectable *gus* expression are illustrated. Ni total four promoters were tested: MAS, Ubi1, Hsp70A/RBCS2, and RBCS2. **C- A.t.+**: negative control, seedlings were co-cultivated with *A. tumefaciens* containing no binary plasmid; **C- A.t.-**: second negative control, seedlings were co-cultivated in media without *A. tumefaciens*.

The Hsp70A/RBCS2_*gus* and the MAS_*gus* promoter constructs exhibited the highest percentage of transformed seedlings with a transformation efficiency of 95 % and 86 %, respectively. The lowest transformation rates were observed with the Ubi1_*gus* and RBCS2_*gus* promoter constructs with 67 % and 58 %, respectively.

(B) GUS stain intensity

The extent of *gus* expression per seedling is represented by the GUS staining intensity and is a critical measure of promoter strength. As the blue staining intensifies in the seedling, the more efficient or stronger the promoter is at driving the expression of the *gus* gene.

The GUS staining intensity was evaluated visually. Seedlings were assigned into five groups from no *gus* expression (Group 0) to significant regions of dark GUS staining (Group 4; Figure 5.8).

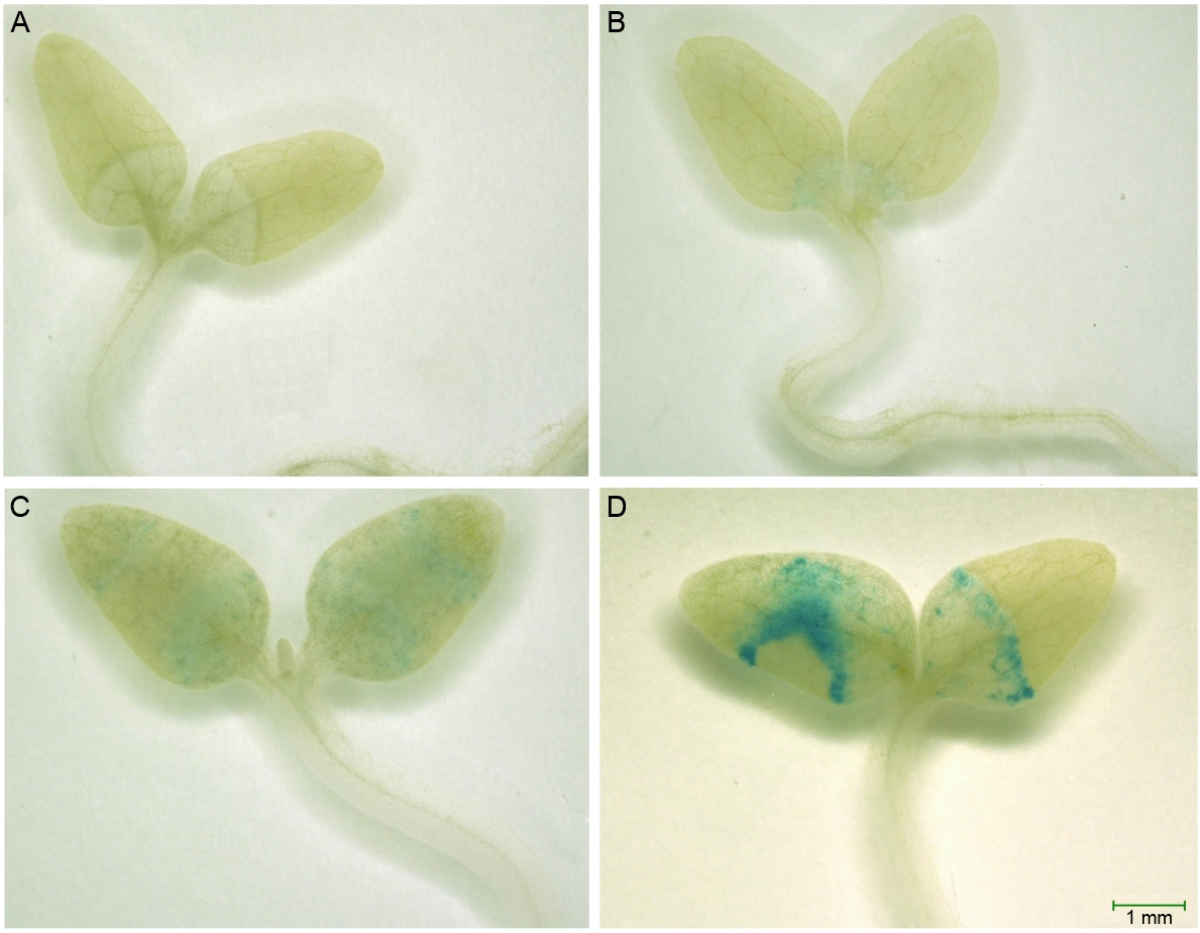

Figure 5.8 | Overview of GUS staining intensities in *N. benthamiana* seedlings. For evaluating promoter strength, seedlings representing four levels of GUS staining were classified into 5 groups. Group 0: seedling showing no GUS activity (not shown), (**A**) Group 1: any light blue GUS staining. (**B**) Group 2: light blue GUS staining, (**C**), Group 3: clearly visible blue GUS staining, (**D**) Group 4: significant regions of dark blue GUS staining.

For evaluation, the percentages of seedlings assigned to the respective groups for each *promoter_gus* construct are summarized in Figure 5.9.

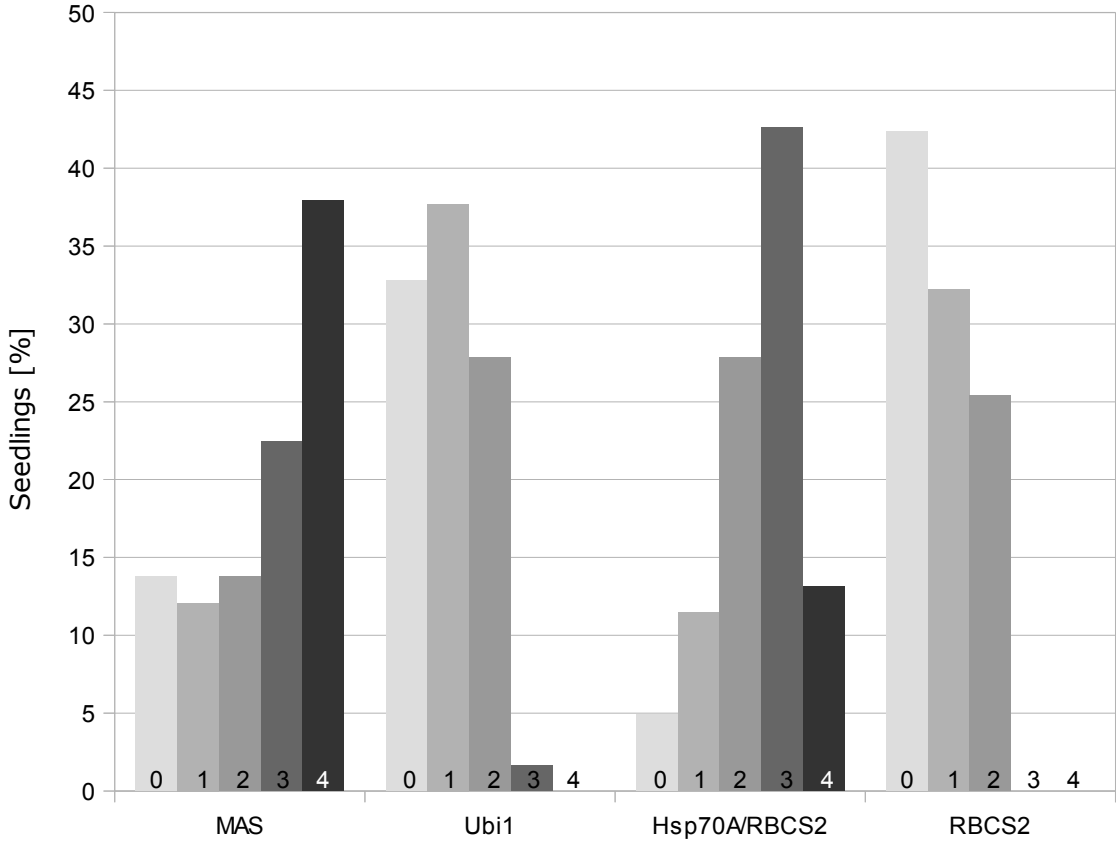

Figure 5.9 | Evaluation of promoter strength in *N. benthamiana* seedlings. The GUS staining intensity reveals the respective promoter efficiency in driving the *gus* gene in *N. benthamiana*. The promoters tested were the MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 promoter within the vector pMDC162. The seedlings were assigned into one of five groups representing increasing levels of GUS staining intensity. Group **0**: seedling showing no GUS activity, group **1**: any light blue GUS staining, group **2**: light blue GUS staining, group **3**: clearly visible blue GUS staining, group **4**: significant regions of dark blue GUS staining (also see Figure 5.9). Shown is the percentage of seedlings per group and per condition.

A majority of the *N. benthamiana* seedlings transformed with the MAS_*gus* construct were classified to either group 4 (38 %) or group 3 (22 %), resulting in more than 60 % of the seedlings showing the highest GUS staining intensities. Only 14 % of the seedlings showed no GUS activity. No other *promoter_gus* constructs showed such a high *gus* expression level.

When the seedlings were transformed with the Hsp70A/RBCS2_*gus* construct, a majority of the seedlings were classified to either group 4 (13 %) or group 3 (43 %), resulting in 55 % of the seedlings showing the next strongest level of GUS expression. Only 5 % of the seedlings showed no *gus* expression.

The lowest expression levels were observed with the RBCS2 and Ubi1 promoter. With the RBCS2 promoter system, the majority of the seedlings (42 %) showed no detectible GUS activity and only a few seedlings with group 3 or 4 GUS expression intensities. Similarly, transformation with the Ubi1 promoter system resulted in 33 % showing no GUS activity and no seedlings with group 3 or 4 GUS intensities.

Conclusion of promoter test in N. benthamiana

In *N. benthamiana* seedlings, the MAS and the Hsp70A/RBCS2 promoter systems resulted in the highest transformation efficiency and the strongest GUS staining intensities of the four *promoter_gus* constructs. The Ubi1 and the RBCS2 promoter systems resulted in significantly lower GUS expression intensities. Based on staining intensity, the MAS promoter showed the strongest expression followed by the Hsp70A/RBCS2 promoter (Figure 5.9).

The strength and efficiency of the MAS promoter in driving recombinant genes in *N. benthamiana* is supported by previous studies. Guevara-Garcia *et al.* (1999) investigated the role of cis-regulatory elements of the MAS promoter driving *gus* in stable transgenic tobacco plants (*N. tabacum*). High expression levels of the *gus* and luciferase genes were observed with the MAS promoter in tobacco plants (Guevara-Garcia *et al.* 1999; Langridge *et al.* 1989). The MAS promoter drove the expression of the luciferase gene in tobacco calli and root tips but only barely in the internods (Langridge *et al.* 1989). In our transiently transformed *N. benthamiana* seedlings, GUS activity was mainly detected within the cotyledons with low or non-existent expression levels in the hypocotyl or root. Recently, *Catharanthus roseus* seedlings (dicotyledon) transiently transformed using the FAST-method also showed strong gus expression with the MAS promoter in a majority of the seedlings (Weaver *et al.* 2014).

To our best knowledge, the expression efficiency of the Hsp70A/RBCS2 and the RBCS2 promoters have not been reported in tobacco. We found RBCS2 to be a relatively weak promoter in driving *gus* in *N. benthamiana* but a strong promoter when fused downstream to the Hsp70A promoter. This was similarly observed in the microalgae *C. reinhardtii* (Schroda *et al.* 2000). The Hsp70A/RBCS2 promoter system has been successfully used in this model organism (Strenkert *et al.* 2013; Neupert *et al.* 2012; Eichler-Stahlberg *et al.* 2009).

The expression levels obtained with the Ubi1 promoter driving the *gus* gene in *N. benthamiana* were very low, but explicable. First, the Ubi1 promoter is commonly used for strong expression in monocotyledonous plants (Gurr and Rushton 2005; Christensen *et al.* 1992) and relies on trans-acting factors present in monocotyledonous plants but lacking in dicotyledons (Christensen *et al.* 1992). Interestingly, the Ubi1 promoter was highly-efficient for expressing foreign genes in *Chlorella* cells (Chen *et al.* 2001; Taylor *et al.* 1993). As a result, the Ubi1 promoter will be evaluated in microalgae despite the results obtained in *N. benthamiana*.

Promoters need regulating elements or introns, which can operate as activators or regulators to enhance or silence the expression (Guevara-Garcia *et al.* 1999; Christensen *et al.* 1992), especially inducible promoters. Trans-acting factors in the host organism may not be present to drive the recombinant gene. The best example is the lack of trans-acting factors in driving Ubi1 promoter expression in dicotyledonous plants. In conclusion, the MAS followed by the Hsp70A/RBCS2 are the most efficient promoter systems in driving the *gus* gene in *N. benthamiana* and are promising candidates for evaluation in *C. vulgaris*.

5.3.1.2.2. Promoter Evaluation in *C. reinhardtii*

Next, the promoter constructs (MAS, Ubi1, Hsp70A/RBCS2, and the RBCS2 promoters driving *gus* gene) were evaluated in the model organism *C. reinhardtii*, a green microalgae like *C. vulgaris.* Several stable transformation strategies are routinely used in *C. reinhardtii*, including the glass bread method (Kindle 1990), PEG-method, silicon carbide whiskers (Dunahay 1993), electroporation (Shimogawara *et al.* 1998), and particle bombardment (Mayfield and Kindle 1990).

For evaluating the promoter strengths in *C. reinhardtii*, the PEG-method was investigated since this method successfully transformed *C. reinhardtii* (Kindle 1990) and also several *Chlorella* species (Huang *et al.* 2006; Kim *et al.* 2002; Hawkins and Nakamura 1999; Jarvis and Brown 1991).

The transformation efficiency (A) and the intensity of the GUS staining in the cells (B) was monitored visually by microscopy.

(A) transformation efficiency

We could not accurately determine the transformation efficiency as the seed cultures were contaminated with fungi. Accurate counting of microalgae cells was not possible since the removal of fungi resulted in the loss of algae cells adhered to the fungal mycelium. However, GUS stained C. *reinhardtii* cells were detected in all four promoter constructs, except for the two negative controls. The negative controls were microalgae transformed with and without the pMDC162_w/oGWsys vector and no GUS staining was observed and once algae transformed without plasmid (Figure 5.10).

As both negative controls, *C. reinhardtii* cells transformed with an unarmed or without plasmid, showed no level of *gus* expression (Figure 5.10). As conclusion, all detectable GUS staining detected in the cells were archived by heterologous expressed *gus*. The GUS-assay was previously tested to work in *C. reinhardtii* by Kumar *et al.* (2004) and Ishikura *et al.* (1999).

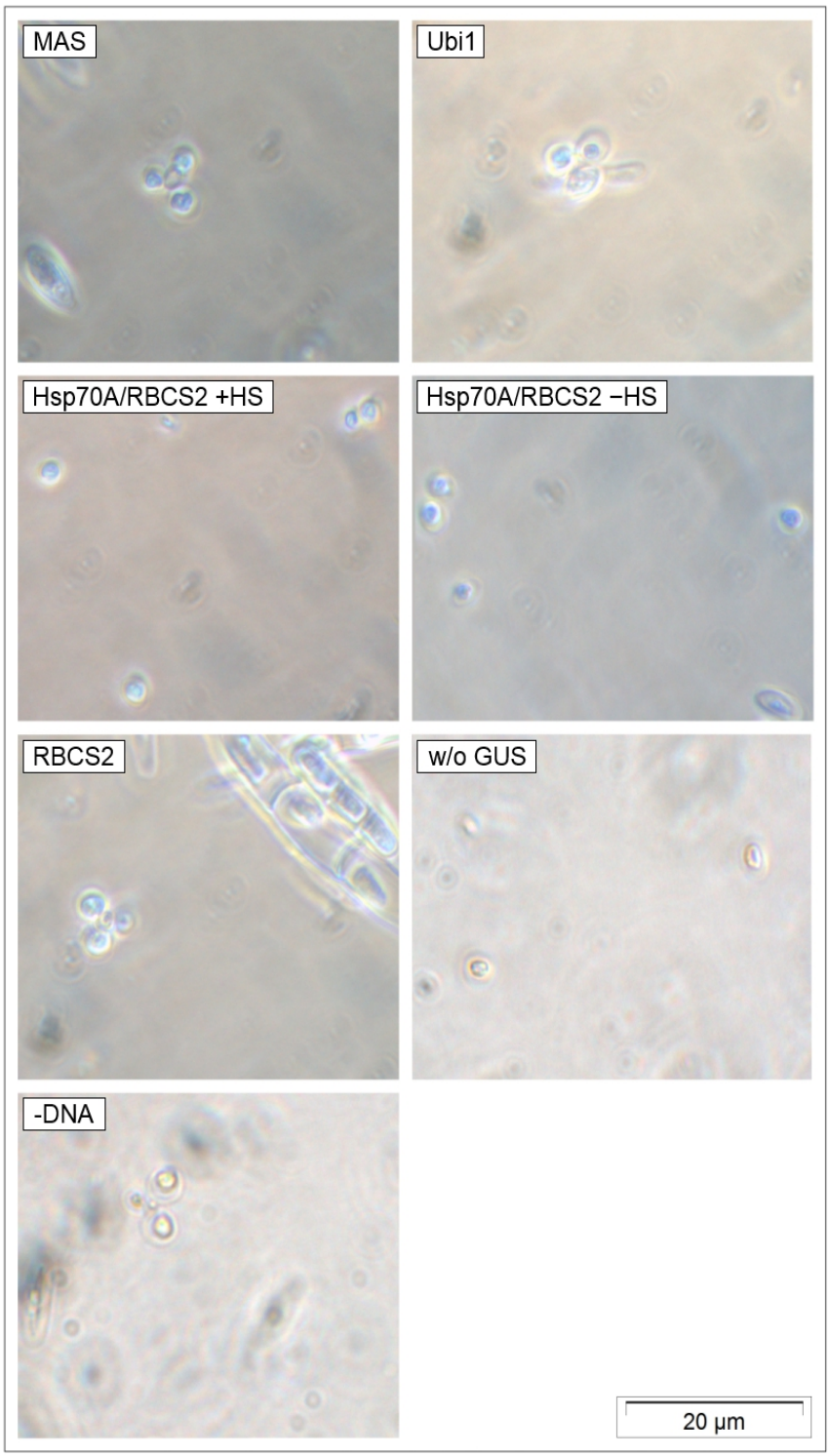

Figure 5.10 | *C. reinhardtii* transformants expressing *gus*. *C. reinhardtii* cells were transformed using the PEG-method (Section 5.2.5.). After cultivation with the *promoter_gus* constructs for 30 hours, the GUS-assay were performed (Section 5.2.4.). Four pMDC162_w/oGWsys_*promoter_gus* plasmids were tested containing either the MAS, Ubi1, Hsp70A/RBCS2, or RBCS2 promoter (Section 5.2.2.). Microalgae transformed with the Hsp70A/RBCS2_*gus* constructs were either (1) Hsp70A/RBCS2 +HS: heat shocked for 40 min at 40 °C and equilibrated for one hour at room temperature before starting the GUS-assay, and (2) Hsp70A/RBCS2 -HS: untreated. The negative controls consisted of microalgae incubated with and without the pMDC162 vector lacking a *promoter_gus* construct (w/o GUS; -DNA).

(B) GUS stain intensity

The promoter strength achieved in *C. reinhardtii* was also assessed by GUS stain intensity. For evaluating GUS stain intensity, each cell was classified in one of three groups, indicating no *gus* expression (Group 0) to significant dark GUS stain (Group 2; Figure 5.11).

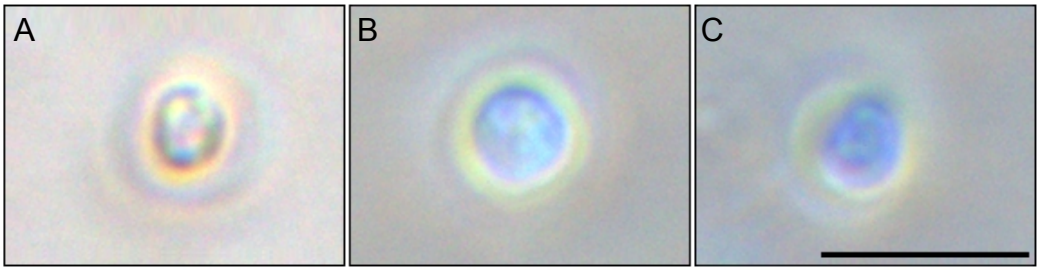

Figure 5.11 | Overview of three GUS stain intensity groups for promoter evaluation in *C. reinhardtii*. Represented are one member each for the three groups in which the cells were assigned to for promoter evaluation. If a cell showed no GUS activity it was classified to group 0 (**A**). If a light blue stain was detectable it was assigned to group 1 (**B**), and when the cell is dark blue it was assigned into group 2 (**C**). The scale bar represents 5 µm.

Due to fungal contamination of the *C. reinhardtii* cultures, only a few cells were evaluated: 30 cells for MAS, 28 cells for Ubi1, 53 cells for Hsp70A/RBCS2 treated with heat (+HS), 38 cells for the untreated Hsp70A/RBCS2 (-HS), and 37 cells for the RBCS2_*gus* construct. For the two negative controls, 32 unstained cells were found when transformed with an unarmed vector and 14 unstained cells were found when no DNA was added for the transformation.

The assignment to the three groups were made by visual performance. The results are shown in Table 5.2 and graphically in Figure 5.12.

Table 5.2 | Evaluation of promoter strength in *C. reinhardtii* cells.

Promoter	Intensity group		
	0	**1**	**2**
MAS	6.7	53.3	40.0
Ubi1	17.9	42.9	39.3
Hsp70A/RBCS2 +HS	3.8	64.2	32.1
Hsp70A/RBCS2 -HS	2.6	44.7	52.6
RBCS2	5.4	83.8	10.8
neg. control -*gus*	100	-	-
neg. control -DNA	100	-	-

The GUS stain intensity reveals the respective promoter efficiency in driving the *gus* gene in *C. reinhardtii*. Tested were the MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 promoter within the vector pMDC162_w/oGWsys. Each cell became assigned to one of three groups representing GUS stain intensities. Group **0** indicates cells with no GUS activity. All *C. reinhardtii* cells classified in group **1** showed a light blue stain. Group **2** includes transformed cells with a dark blue stain (also see Figure 5.12). Shown is the percentage of evaluated cells per condition. The negative controls contain the transformation with an unarmed plasmid (-*gus*) and without plasmid (-DNA).

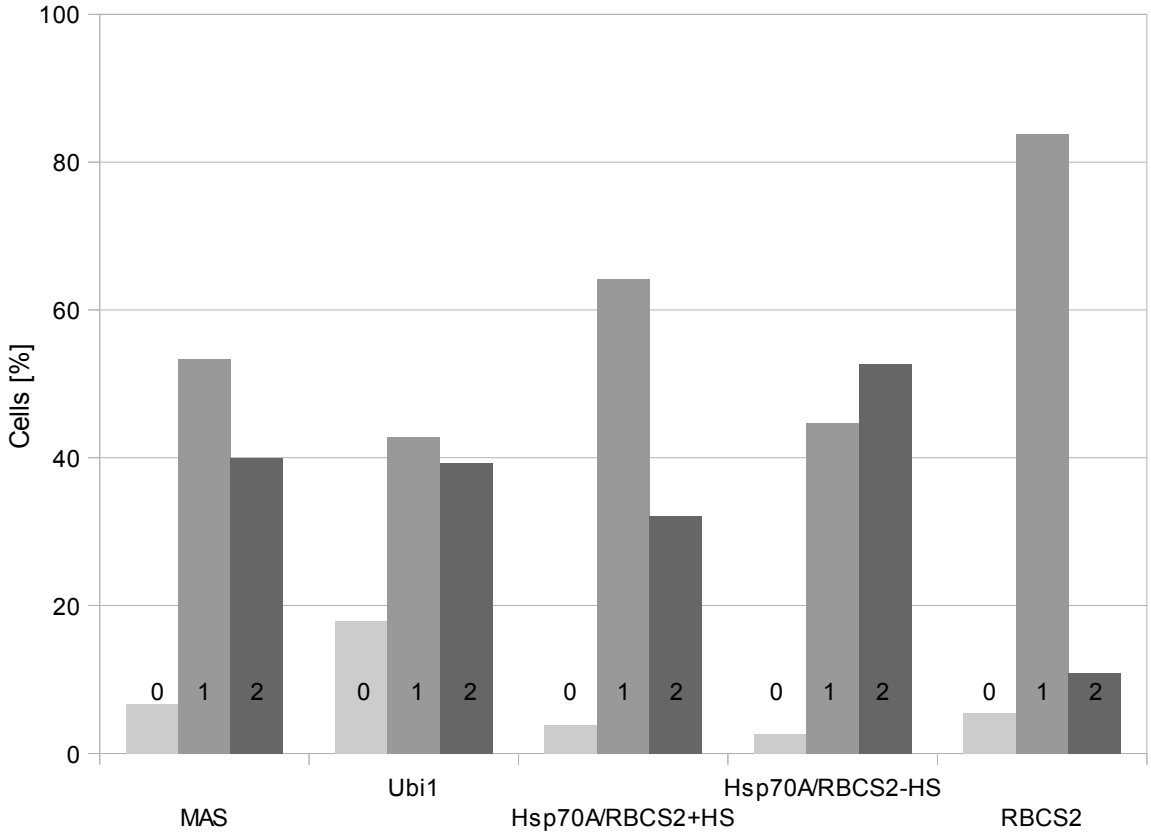

Figure 5.12 | Evaluation of promoter strength in *C. reinhardtii* cells. The GUS stain intensity reveals the respective promoter efficiency in driving the *gus* gene. Tested were the MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 promoter within the vector pMDC162_w/oGWsys. The cells were assigned into one of three groups representing GUS stain intensities. Group **0** indicates cells with no GUS activity. All *C. reinhardtii* cells classified in group **1** show a light blue stain. Group **2** includes transformed cells with a dark blue stain (also see Figure 5.12). Shown is the percentage of evaluated cells per condition.

In *C. reinhardtii*, the uninduced cells transformed with the Hsp70A/RBCS2 construct (-HS) showed the highest *gus* expression level with approximately half of the cells assigned to group 1 (45 %) and group 2 (52 %). Only 3 % of the cells did not show any level of *gus*. In contrast, a majority of the cells transformed with the Hsp70A/RBCS2 construct and treated with heat shock were assigned to group 1 (64 %) and only 32 % to group 2. About 4 % of these cells showed no *gus* expression. The strength of the MAS promoter was similar to the heat-shocked Hsp70A/RBCS2 promoter, with 53 % classified in group 1, 40 % in group 2, and 7 % unstained. Interestingly, *gus* expression was observed with the Ubi1 promoter in *C. reinhardtii* with approximately 40 % of the cells classified in group 1 and 2.

Conclusion of promoter test in C. reinhardtii

In *C. reinhardtii*, the strongest promoter in driving the *gus* gene within the pMDC162_w/oGWsys vector was the Hsp70A/RBCS2 in the absence of heat shock and the MAS promoters. These results were similar to that obtained with the *N. benthamiana* seedlings. As previously observed, the lowest level of *gus* expression was observed with the RBCS2 promoter. A surprise was the expression level when *gus* was driven by the Ubi1 promoter. In contrast to its expression in *N. benthamiana*, the expression level of *gus* using the Ubi1 promoter in *C. reinhardtii* increased and is comparable to that with the MAS or the heat shocked Hsp70A/RBCS2 promoters. Our result indicates that *C. reinhardtii* produces trans-acting factors similar or at least partial functional to those produced by monocotyledons.

As previously reported, the RBCS2 promoter exhibits low efficiency when acting alone compared to those fused to another promoter such as Hsp70A (Strenkert *et al.* 2013; Schroda *et al.* 2000), the CaMV 35S promoter (Hawkins and Nakamura 1999), or in combination with the RBCS2 intron 1 (Lumbreras *et al.* 1998). The RBCS2 promoter alone was not efficient enough for applications of recombinant gene expression in *C. reinhardtii* (Strenkert *et al.* 2013; Schroda *et al.* 2000; Hawkins and Nakamura 1999; Lumbreras *et al.* 1998). Currently, the Hsp70A/RBCS2 promoter combination seems to be the most efficient chimeric promoter system for *C. reinhardtii* and is the most frequently used promoter for recombinant gene expression in this organism (Strenkert *et al.* 2013; Eichler-Stahlberg *et al.* 2009). Surprisingly, the heat shock treatment previously described by Schroda *et al.* (2000) did not increased *gus* expression in our experiment and instead slightly decreased *gus* expression. Schroda *et al.* (2000) reported about an increase in *gus* expression 60 - 90 min after treatment. Possibly, the effect could be obtained, when the time before starting the GUS-assay and evaluation become extended from one to two hours.

In conclusion, the Hsp70A/RBCS2, MAS, and Ubi1 promoters are strong candidates for driving recombinant gene expression in *C. vulgaris*. As expected, the RBCS2 promoter alone is not efficient enough compared to the other promoters tested. The Hsp70A/RBCS2 and the Ubi1 promoters are also functional in other microalgae species besides *Chlamydomonas* and *Chlorella* (Li and Tsai 2009; Chen *et al.* 2008; Chen *et al.* 2001). As the MAS promoter was not applied for recombinant gene expression in microalgae, further investigations about its promoter strength in *C. vulgaris* would be preferable.

5.3.2. Transformation of *C. vulgaris*

The transformation turned out to be the most challenging step of this work. In this section, we describe the genetic engineering attempts to stably transform *C. vulgaris* cell with the pCAMBIA1305.1_hygB_MAS_GUS plasmid (Chapter 3, Section 3.1.9., Table 3.8) or our previous constructed plasmids (Section 5.2.2.). We tested the methods of electroporation, *Agrobacterium*-mediated transformation, and the PEG method, described in the following.

5.3.2.1. Electroporation

Electroporation can easily transform protoplasts, cell-wall-deficient mutants, or cells with thin walls without any special treatment (Chow and Tung 1999). Electric pulses render the cell membrane permeable for a short time so that the DNA can enter the cells (Hallmann 2007). Electroporation has successfully transformed different *Chlorella* species (Huang *et al.* 2006; Chen *et al.* 2001; Chow and Tung 1999; Maruyama *et al.* 1994).

We analyzed and optimized the electroporation method for *C. vulgaris*. First, we determined the survival rate of cells treated with different field strength, without DNA and selection pressure. In a second experiment, we tested different voltage in the presence of DNA and selected on hygB containing media plates to establish the right conditions for a high transformation efficiency.

To determine the survival rate, cell suspensions were electroporated under three different field strengths (1500 V/cm, 1800 V/cm, and 2000 V/cm) according to the protocol in Chow and Tung (1999) and then plated on non-selective media. Cell suspensions plated on non-selective plates served as the positive controls, whereas those plated on hyg B containing media served as the negative controls. The results of the survival experiment are shown in the following (Figure 5.13).

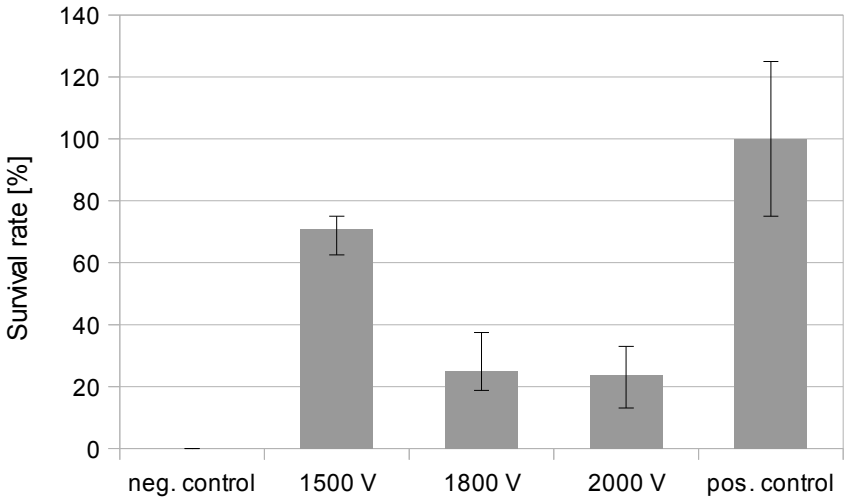

Figure 5.13 | Cells survival rate after exposure to different field strengths. Cell suspensions were electroporated with three different field strengths (1500 V/cm, 1800 V/cm, 2000 V/cm) and plated on non-selective media. Cell suspensions plated on non-selective media served as the positive control. Cell suspensions plated on hygB containing media plates served as the negative control. Evaluation was performed 26 days after plating. The amount of electroporated cells grown was normalized with respect to the positive control (100 %) and the negative control (0 %). The experiment was performed with three biological replicates.

The survival rate of cells after treatment with an electric pulse strength of 1500 V/cm was 71 % in respect to the positive control. Increasing the field strength to 1800 V/cm and 2000 V/cm decreased the survival rate dramatically to 25 % and 24 %, respectively.

In the second experiment, we determined the transformation efficiency of *C. vulgaris* electroporated in the presence of DNA at three different electric field strength. The pCAMBIA1305.1_hygB_MAS_GUS (Chapter 3, Section 3.1.9., Table 3.8) was mixed with *C. vulgaris* suspensions before electroporation at a field strength of 1500 V/cm, 1800 V/cm, and 2000 V/cm. After electroporation the cell suspensions were plated on hyg B containing media plates. The evaluation was performed 30 days after electroporation (Figure 5.14).

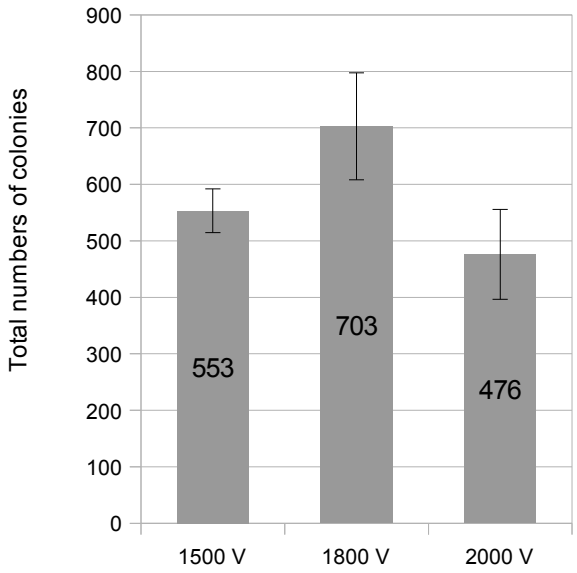

Figure 5.14 | Number of colonies resistant for selection on hygB containing media plates after electroporation with three different field strengths. *C. vulgaris* cells were transformed with the plasmid pCAMBIA1305.1_hygB_MAS_*gus*. Evaluation 30 days after transformation.

The highest number of *C. vulgaris* colonies on the selective media (an average of 703 colonies) resulted when the cells were exposed to an electric pulse strength of 1800 V/cm. This result is significantly higher than for cells electroporated with a field strength of 1500 V/cm and 2000 V/cm resulting in an average total amount of 553 and 476 colonies, respectively.

Although the survival rate is relatively low with electroporation at a field strength of 1800 V/cm (25 %; Figure 5.13), the transformation efficiency is higher than when it were performed at a field strength of 1500 V/cm or 2000 V/cm. Chow and Tung (1999) also performed electroporation at these three different field strengths (1500 V/cm, 1800 V/cm, 2000 V/cm) for *C. vulgaris* and observed the highest number of colonies at a field strength of 1800 V/cm (average 549 colonies). Other electroporation protocols with *Chlorella* also applied a field strength of 1800 V/cm (Huang *et al.* 2006; Chen *et al.* 2001). As a conclusion, we will use a field strength of 1800 V/cm for *C. vulgaris* transformation in further experiments.

5.3.2.2. Agrobacterium-mediated Transformation[1]

In the *Agrobacterium*-mediated method, microalgae were co-cultivated with *A. tumefaciens* (Section 5.2.5.). The *A. tumefaciens* cells contained the constructs for integration into the algal genome. The gene transfer of the cells occurred in a naturally way. The first report about a stable genome transformation of microalgae by applying the *Agrobacterium*-mediated gene transfer was reported by Kumar *et al.* in 2004 for *C. reinhardtii*. Furthermore, they determined a high transformation efficiency for this technique. The method had further be optimized for *C. reinhardtii*, recently (Pratheesh *et al.* 2014). In 2012, also a protocol for *C. vulgaris* resulting in a transformation efficiency of up to 25 % were published (Cha *et al.* 2012). To establish this transformation method for *C. vulgaris*, we used the previously compiled construct pMDC162_Hsp70A/RBCS2_*gus* (Section 5.2.2.).

The experiment were evaluated via the *gus* reporter gene, two days after co-cultivation. The cells were clearly blue after the GUS-assay was performed. In the no-plasmid control no blue color was detectable (Figure 5.15).

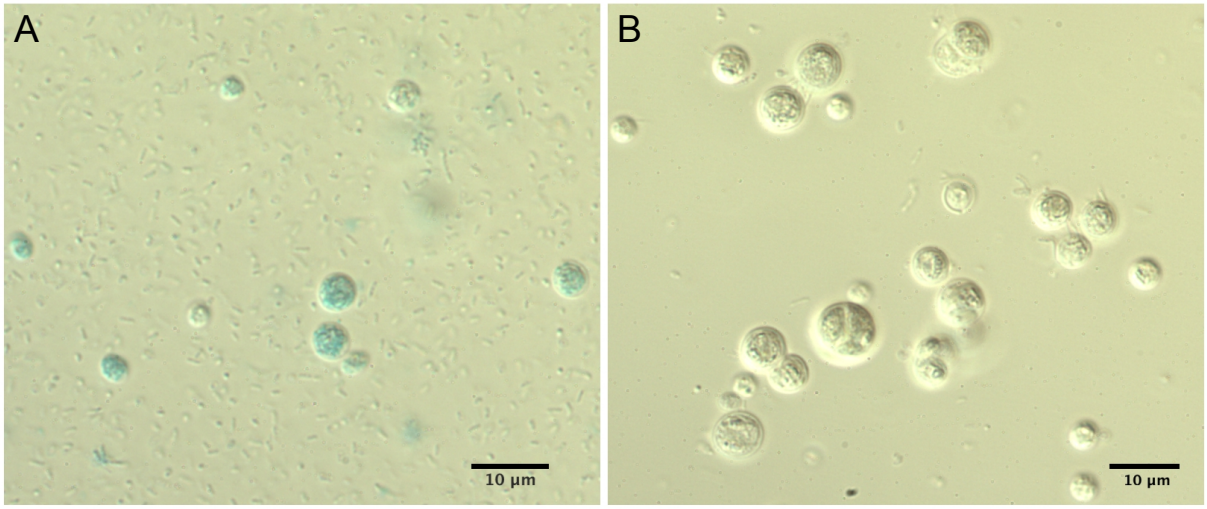

Figure 5.15 | *C. vulgaris* transformants expressing *gus*. *C. vulgaris* cells were transformed using the *Agrobacterium*-mediated gene transfer. After co-cultivation with *A. tumefacience* the approach were incubated for two days before performing the GUS-assay. **A**, co-cultivation with *A. tumefacience* containing the plasmid pMDC162_w/oGWsys_Hsp70A/RBCS2_*gus*. One hour before the GUS-assay were performed, the cells were heat shocked for 40 min at 40 °C. **B**, negative control, co-cultivation with *A. tumefacience* containing the pMDC162_w/oGWsys vector without a *promoter_gus* construct.

The blue stain is clearly visible, when *C. vulgaris* were co-cultivated with *A. tumefaciens* containing the *promoter_gus* construct. Unfortunately, the *gus* gene were also expressed by *Agrobacterium*. This could cause the algae to turn blue when stained giving false positive. Another indication for false positive blue algal cells were that they never grew on selective media. As a solution the *gus* gene needs an intron, so that the *Agrobacterium* cannot express the *gus* gene and positive transformants are clearly detectable.

1 This experiment was performed by Alison Wirshing, PhD student at NEU.

5.3.2.3. PEG-Method

The PEG transformation method requires the removal of the cell wall or using cell-wall-deficient strains available for *C. reinhardtii*. Protoplasts are able to take up DNA when cultivated in the presence of PEG. This transformation technique resulted in the stable transformation of several *Chlorella* species, such as *C. ellipsoidea* (Jarvis and Brown 1991; Kim *et al.* 2002), *Chlorella* sp. DT (Huang *et al.* 2006), *C. sorokiniana*, and *C. vulgaris* (Hawkins and Nakamura 1999).

For the transformation of *C. vulgaris,* we used the protoplast preparation and transformation protocol according to Kim *et al.* 2002 (Section 5.2.5.). The pMDC162_w/oGWsys vectors contained the MAS_*gus*, Ubi1_*gus*, Hsp70A/RBCS2_*gus*, and RBCS2_*gus* constructs (Section 5.2.2.). The unarmed pMDC162_w/oGWsys plasmid (without *gus*) and an approach were no DNA was added served as the negative controls. None of these conditions showed any level of GUS staining.

There are several possible reasons why the transformation of *C. vulgaris* failed. First, the culture was already in the late stationary phase and contained about 4 x 10^8 cells/mL. Therefore, it is likely that the enzyme concentration was too low for efficient protoplast production. In total we used 50 mL of the *C. vulgaris* culture which contained 2 x 10^{10} cells in total, which was twice that of Kim *et al.* (2002) at 5 - 10 x 10^9 cells. In *C. reinhardtii* we performed the same transformation technique with success (Section 5.3.1.2.2.), but with lower cell density (harvested culture 2 x 10^7 cells/mL; 10^9 cells in total for transformation). Second, the cell wall of *Chlorella* species has been reported to be thick and tough (Radakovits *et al.* 2010; Huang *et al.* 2006), which influences the digestion efficiency and results in low protoplast quality.

Both factors, high cell density and the tough cell wall construction of *C. vulgaris* together, resulted in the failed transformation. In future approaches, a lower cell density should be considered.

5.4. *Conclusion*

In this study, we successfully constructed a highly-efficient expression system within the vector pMDC612 and tested several transformation methods to achieve a comprehensive and efficient genetic engineering protocol for *C. reinhardtii.* This genetic engineering protocol can be adapted to *C. vulgaris* for overexpressing potential *goi´*s easily, rapidly, and with high impact.

In summary, the construction of highly-efficient expression cassettes containing several promoters were evaluated with the *gus* reporter gene in *N. benthamiana* and *C. reinhardtii*, including the MAS, Ubi1, Hsp70A/RBCS2, and RBCS2 promoter. All promoters, including MAS, which was never tested in microalgae before, worked in microalgae and in dicotyledonous plants. The MAS as well as the Hsp70A/RBCS2 resulted in the strongest expression in both species. The Ubi1 promoter drives weak expression in the dicotyledon *N. benthamiana,* but resulted in similar expression level as the MAS and Hsp70A/RBCS2 promoter in *C. reinhardtii*. In both experiments, the RBCS2 drove the weakest expression. As the transformation is being repeated in *C. reinhardtii*, the final expression cassettes containing the *goi* driven by all four promoters within the pMDC162 vector were constructed. The final expression cassettes contain a highly-efficient 5´ - and 3´ - UTR, tags for identification and detection of

the protein (flag-, strepII-, and 6x his-tag), and the *goi*, *Cvudgat1*. In conclusion, we created four vectors to investigate every potential *goi* via efficient overexpression analysis by a simple exchange of the gene within this high-powered expression cassettes.

To establish a transformation method for *C. vulgaris* we tested electroporation, *Agrobacterium*-mediated gene transfer, and transformation via PEG-method. For electroporation we determined the optimal field strength of 1800 V/cm to successfully transform *C. vulgaris* cells. Unfortunately, the cells which grew on the selective plates were never tested. The success of the *Agrobacterium*-mediated transformation was unclear as the *Agrobacterium* was also transformed with the *Gus* gene so transformation efficiency of the *C. vulgaris* could not be distinguished. The transformation of *C. vulgaris* cells with the PEG-method was successful but needs further optimization of the cell density for protoplast production.

In conclusion, effective transformation protocol for *C. vulgaris* appears positive and will require further optimization, which we were not able to implement in this the study. This was mainly caused as the this work was distributed to two locations, at Northeastern University Boston (NEU; USA) and Leibniz University Hanover (LUH; Germany). All transformation work were performed at NEU and the time in Boston was limited by visa reglementations. This obstructed the work flow several times and caused the non-achievement for this objective.

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6. Conclusion and Prospects

In this thesis, we focused on improving the lipid productivity of the microalgae *Chlorella vulgaris* (Beyernick 1890) through genetic engineering. We chose *C. vulgaris*, as several studies revealed this microalgae to be a good biodiesel producer (Mallick *et al.* 2012; Kurt 2011; Converti *et al.* 2009; Illman *et al.* 2000), with a desirable biodiesel profile (Praveenkumar *et al.* 2012; Hobuss *et al.* 2011; Guarnieri *et al.* 2011; Kurt 2011; Zheng *et al.* 2008; Rashid *et al.* 2008).

The overall goal of this thesis research was to overcome the potential bottleneck of triacylglycerol (TAG; oil) production, identified to be diacylglycerol acyltransferase type-1 (DGAT1), by genetically engineering the microalga *C. vulgaris*. In the **first aim**, we identified and characterized DGAT1 from the microalgae *C. vulgaris* (CvuDGAT1). Characterization involved identification of DGAT1 motifs, transmembrane domains, and proposed tertiary structure of the DGAT1 protein through bioinformatic tools. Then, we functionally characterized the *dgat1* gene in non-oil-producing yeast strains through the recovery of oil-production with *dgat1* expression. In the **second aim**, we established a complete genetic engineering protocol for *C. vulgaris*, to provide an easy and rapid protocol for overexpression or impact studies of a *gene of interest* (*goi*). This includes the construction of a highly-efficient, tailor-made expression cassette for *C. vulgaris,* and the optimization of a suitable and efficient transformation method for this alga. Our desired goal was to verify the genetic engineering protocol by expressing the *Cvudgat1* gene and investigating its impact on oil productivity in *C. vulgaris*.

Aim 1: We identified and characterized DGAT1 from the microalgae *C. vulgaris*

DGAT1 is one of three types DGATs, DGAT1 to DGAT3, which are involved in oil-biosynthesis (Kennedy 1961). DGAT1 and DGAT2 catalyze the same reaction, which is the last step in TAG-production (Chen and Smith 2012; La Russa *et al.* 2012; Lung and Weselake 2006). Every species have the preference for one of these two isoforms (Shockey *et al.* 2006). In several studies, the DGAT1 protein was determined as limiting enzyme for TAG-production in *C. vulgaris* (Guarnieri *et al.* 2011; Miller *et al.* 2010).

We successfully identified the *dgat1* gene from *C. vulgaris* (*Cvudgat1*) by constructing a gDNA and cDNA library with the GenomeWalker™ Universal Kit and the SMARTer™ RACE cDNA Amplification Kit (Clontech), respectively. Then, we applied these libraries in nested-PCRs to decode *Cvudgat1*. The *Cvudgat1* gene has a gDNA size of 2,854 bp and a cDNA size of 1,383 bp. The corresponding protein sequence (CvuDGAT1) consists of 460 aa (amino acids) with a molar weight of 53.5 kDa.

Bioinformatic analysis revealed that CvuDGAT1 shares typical motifs of DGAT1 proteins from plants and microalgae. These include: the binding domains for the two substrates acyl-CoA and diacylclygerol (DAG), the putative active site, the highly conserved fatty acid (FA) motif and the ER-retrieval motif at the C-terminal end. Additionally, we found the highly conserved phenylalanine, which was determined to have a high impact on DGAT1 activity (Zheng *et al.* 2008). DGAT1 is a member of the membrane bound O-acyltransferase (MBOAT) superfamily. Those proteins are characteristically integrated into the ER membrane with eight to ten transmembrane domains (TMDs). MBOAT proteins

transfer organic acids, mostly FAs, onto hydroxyl groups of membrane-embedded target molecules (Hofmann 2000). We identified nine hydrophobic regions for CvuDGAT1, as predicted by six different bioinformatic tools. Additionally, the putative tertiary structure of DGAT1 and its embedding into the ER membrane was elucidated with two tertiary structure prediction tools. The predicted protein and its membrane integration appeared feasible and suitable with regard to its catalyzing activity and the availability of binding domains for their substrates. The active site and the phenylalanine are located on the surface of the DGAT1 and adjacent to each other. Therefore, our CvuDGAT1 model meets the requirements for the catalyzing the attachment of acyl-CoA and DAG to TAG.

Next, we functionally characterized *Cvudgat1* by heterologous expression in a quadruple disrupted non-oil-producing yeast mutant. We visualized the non-polar lipids via the stain Bodipy® 493/503 and determined the relative oil-content of these yeast strains by flow cytometry. We found the expression of *Cvudgat1* in the non-oil-producing yeast strain restored its capability to synthesize TAGs. This result demonstrated that the corresponding protein of the gene isolated from *C. vulgaris* has DGAT enzyme activity in yeast and that it is involved in TAG-biosynthesis.

Aim 2: We constructed a highly-efficient, tailor-made expression system for *C. vulgaris* in a suitable vector and tested several transformation methods to establish a complete and efficient genetic engineering protocol for *C. vulgaris*

We chose the pMDC162 vector, which is derived from the pCAMBIA T-DNA cloning vectors (Curtis and Grossniklaus 2003). pCAMBIA vectors are commonly applied in a range of plant species[1] and newly also in microalgae (Kumar *et al.* 2004). We chose this vector as the T_i-region already contains the chosen hygromycin B resistance gene (*hptII*) for selection of transformants and suitable restriction sites for insertion of the final expression cassettes. In the backbone of the vector, the kanamycin resistance gene (*nptII*) for bacterial selection is present, and two efficient replicons for high reproduction in *E. coli* and *A. tumefaciens*. Additionally, the vector contained the Gateway system and the *gusA* gene, which were removed, as it was not required. This pMDC162 with the removed regions has a reduced vector size of about 9.5 kbp, which made this plasmid easier to handle and a perfect vector for transformation of *C. vulgaris.*

The choice of a suitable promoter is important in constructing a highly efficient expression cassette for recombinant gene expression. In the green alga *Chlamydomonas reinhardtii,* most foreign promoters resulted in poor expression levels (Neupert *et al.* 2012). A common and well-studied promoter fusion for *C. reinhardtii* is the heat shock protein 70A promoter (Hsp70A) fused downstream to the promoter of the small subunit 2 of ribulose-1,5-bisphosphate carboxylase/ oxygenase (RBCS2; Schroda *et al.* 2000).

We tested four different promoters in *Nicotiana benthamiana* and *C. reinhardtii:* the common Hsp70A/RBCS2 chimeric promoter system, the RBCS2 promoter alone, the polyubiquitin-1 (Ubi1) promoter, which showed good expression levels in *Chlorella ellipsoidea* (Chen *et al.* 2001), and the mannopine synthase (MAS) promoter, which has not been applied in microalgae. The promoters were

1 http://www.cambia.org/daisy/cambia/585.html#dsy585_References; Status Sept. 29th 2014.

evaluated via the ß-glucuronidase (*gus*) reporter system. We found MAS and Hsp70A/RBCS2 resulting in the strongest expression in both species. The RBCS2-directed expression was the weakest in both systems. The Ubi1 promoter resulted in a similar expression level as MAS in *C. reinhardtii*, but in the dicotyledon *N. benthamiana,* the expression level was as low as when controlled by the RBCS2 promoter. This can be explained by the absence of trans-acting factors in dicotyledons that are normally found in monocotyledons for which the Ubi1 promoter is usually applied (Christensen *et al.* 1992).
For construction of the final vector, we used the pMDC162 with the removed Gateway system and *gusA* gene and inserted a highly-efficient 5´- and 3´-untranslated region (UTR), the promoter and the *Cvudgat1* gene to obtain the construct pMDC162_w/oGWsys_*promoter_Cvudgat1*. The next intended step was the integration of this vector into *C. vulgaris* and finally analyze its impact on oil metabolism.

The most challenging step for engineering microalgae was the transformation of *C. vulgaris*. The transformation efficiency is strongly species-dependent and needs to be optimized for every microalgae species (Radakovits *et al.* 2010; Hallmann 2007). Commonly used methods are the glass-bead method (Kindle 1990), which is similar to the polyethylene glycol (PEG-) method (Jarvis and Brown 1991), the silicon carbide (SiC) method (Dunahay 1993), electroporation (Maruyama *et al.* 1994), particle bombardment (Mayfield and Kindle 1990), and the *Agrobacterium*-mediated gene transfer (Kumar *et al.* 2004). Several of these methods require prior preparation of protoplasts, including the glass-bead method, the PEG-method and preferably electroporation. For the model organism *C. reinhardtii* cell-wall-deficient mutants are available to circumvent this precarious step. Unfortunately, no cell-wall-deficient strains have been developed for other green algae species (Kim *et al.* 2002), which makes protoplast preparation inevitable.

To find a suitable transformation method for *C. vulgaris* we tested the two most frequent applied transformation methods for *Chlorella*, including electroporation and the PEG-method. Additionally, we performed the *Agrobacterium*-mediated gene transfer.
Electroporation was performed with *C. vulgaris* cells (no protoplasts) and the use of three different field strengths (1500 V/cm, 1800 V/cm, 2000 V/cm). We found the highest transformation efficiency with a field strength of 1800 V/cm. But the engineered algae needed about 30 days to form little colonies. Unfortunately, these colonies were never tested. To improve this method, the growing conditions for the selection have to be adjusted and optimized. In parallel, the work with protoplasts for electroporation should be considered.
The **PEG-method** resulted in blue-stained cells with *C. reinhardtii* (promoter test; see above) but not for *C. vulgaris*. A significant difference between these both approaches was the amount of cells used in protoplast production. For *C. vulgaris* the cell concentration was about 20-times higher than for *C. reinhardtii*. As future work, the transformation via PEG-method needs to be repeated with a lower cell density.

The ***Agrobacterium*-mediated gene transfer** was performed by Alison Wirshing, a PhD student from NEU. The attempts resulted in cells showing clearly blue *gus* expression. Unfortunately, the *gus* gene was also expressed by *Agrobacterium*. This could cause the algae to turn blue when stained giving false positive. Another indication for false positive blue algal cells was that they did not grow on selective media. As a solution, the *gus* gene needs an intron so that the *Agrobacterium* cannot express the *gus* gene and positive transformants are clearly detectable.

The transformation, in general, is the most challenging aspect of engineering microalgae. When considering transformation protocols, standard protocols are only available in the model green algae *C. reinhardtii*. Currently, there are reports of a few species that are routinely transformed, including *Volvox carteri*, the diatom *Phaeodactylum tricornutum,* and several *Chlorella* species (Walker *et al.* 2005; León-Bañares 2004) but generally no follow-up publications are available for species other than *C. reinhardtii*.

The main difficulty for the establishment of the transformation method for *C. vulgaris* was the distribution of the dissertation to two locations, at Northeastern University Boston (NEU; USA) and Leibniz University Hanover (LUH; Germany). All transformation work was performed at NEU and the time in Boston was limited by visa reglementations. This obstructed the work-flow several times, affecting the completion of this objective.

Prospectively, *C. vulgaris* should be transformed with the pMDC162 vector containing the *Cvudgat1* gene within our highly-powered expression cassette. With this, not only the completed genetic engineering protocol can be tested, but also the impact of the overexpression of the *Cvudgat1* gene on TAG-biosynthesis in *C. vulgaris* can be pursued. Additionally, other candidates could be expressed or co-expressed with the *Cvudgat1* gene to investigate if this even increase the oil productivity of *C. vulgaris.*

Recommendation 1:

First, the establishment of an efficient transformation method for stable integration of recombinant DNA is a urgent need. In this thesis, we tried three different and commonly used genetic engineering protocols for *C. vulgaris*. None of them resulted in verified stable transformants.

From our perspective, the most promising method for stable integration into the algal genome represents the *Agrobacterium*-mediated gene transfer. The prior aim of the other discussed methods, including the glass-bead method, the PEG-method, electroporation, particle bombardment, and the SiC-method, is the insertion of the DNA into the cell, but there is no system, which guarantees or increases the likelihood for stable integration. In contrast, behind *Agrobacterium*-mediated transformation stands a naturally occurring process for active integration of DNA into the genome.

As a result, we will concentrate on optimization on the *Agrobacterium*-mediated gene transfer method to stable transform *C. vulgaris*. Therefore, the reporter gene needs an intron, so that the *Agrobacterium* cannot express the *gus* gene and positive transformants are clearly verified by microscopy.

Recommendation 2:

After establishment of an efficient transformation protocol, a first impression about the impact of *Cvudgat1* gene on oil-production should be pursued. Therefore, flow cytometry with cell sorting could be an interesting tool. Flow cytometry can collect cells that match specific criteria. Cells that survive the sorting can be cultivated and propagated afterwards. With this strategy, *Cvudgat1*-transformed *C. vulgaris* cells can be recovered from the plates shortly after transformation, stained with Bodipy® 493/503 and sorted via flow cytometry by emitted light intensity, which corresponds with the algal oil-content. Then, these cells can be cultured as a *high-oil-producing* group and single pure colonies or clones can be obtained for detailed future investigations. For instance,

1) Stable integration can be verified by PCR and southern blot analysis. Quantitative reverse transcriptase (RT-PCR) would determine increases in *dgat1* transcription levels of the engineered *C. vulgaris* clones. The recombinant protein can be detected by immunostaining, as three tags are available (flag-, strepII-, 6x his-tag) at the 3´ -end of the *Cvudgat1* gene. With specific antibodies, the recombinant protein can be identified and its production evidenced for transformed *C. vulgaris* cells.
2) To measure the impact of *Cvudgat1* overexpression on the lipid content of *C. vulgaris*, several methods can be applied, including the transesterification and GC-MS analysis, flow cytometry, and microscopy. Protocols for all three techniques were established and optimized at NEU.

Recommendation 3:

After investigations on the oil-content of overexpressing *Cvudgat1* transgenic *C. vulgaris* lines, it would be even more interesting to overexpress a second gene to further increase oil productivity of this alga. Thereby, an interesting group of candidates are represent transcription factors (TFs) of oil-synthesis. The DGAT1 enzyme can be seen as a "*pull*" factor for TAG-biosynthesis, TFs, thereby, operate as "*push*" factor by up-regulating enzymes involved in TAG-biosynthesis. When a *C. vulgaris* transgenic line with a combined overexpressed "*pull"* and "*push"* factor is generated and an even higher oil-yield can be expected.

We already started investigations in this direction and found in total four potential TFs candidates. All showed high impact in regulation of the oil-biosynthesis in plants. These include the SHOOT MERISTEMLESS (STM; Scofield *et al.* 2013), LEAFY COTYLEDON 1 (LEC1; Lotan *et al.* 1998), LEAFY COTYLEDON 2 (LEC2; Baud et al. 2007) and WRINKLED1 (WRI1; Cernac and Benning 2004) TF. Thereby, STM up-regulates i. a. LEC1 and LEC2 these in turn increase i. a. WRI1 expression. WRI1 itself results in up-regulation of the expression of several genes, which enzymes are involved in FA-biosynthesis. We further investigated, if these higher plant TFs occur also in green algae. Therefore, we used the NCBI pBLAST search in which we inserted the TF protein sequences from *Arabidopsis thaliana* and looked for matches in the model *C. reinhardtii*. Based on conserved domain identities, we found the LEC1, LEC2 and WRI1 are likely present in *C. reinhardti*i, but not the STM TF. We suggest, that the presents of the STM TF in higher plants is based on evolutionary development and therefore not present in green algae.

The combination of DGAT1 and WRI1 overexpression in the higher plant *A. thaliana* resulted in a high increased of its oil-content (Vanhercke *et al.* 2013). Based on this, the overexpression system out of the *dgat1* gene as "*pull"* factor and the *wri1* TF gene as "*push"* factor represents itself as a promising combination to further increase TAG productivity of *C. vulgaris*. In future work, we will identify the *wri1* gene from *C. vulgaris* and characterize its protein similar to the DGAT1 protein in Chapter 4. Then, the two genes could be fused together to a polycistronic gene by the IRES- or the 2A-element, inserted into the optimized pMDC162 vector, and finally co-expressed in *C. vulgaris.*

In summary, we successfully identified and characterized the *dgat1* gene from *C. vulgaris* and demonstrated that CvuDGAT1 has DGAT1 activity and is involved in TAG-biosynthesis. We further found that CvuDGAT1, indeed, could represent the limiting enzyme in oil-production in *C. vulgaris*. To reveal CvuDGAT1 impact in oil synthesis, we constructed a genetic engineering protocol to establish an easy and rapid protocol for overexpression or impact studies of a *goi* in *C. vulgaris*.
In future work, we will pursue three aims to increase *C. vulgaris* oil-productivity: (1) optimization of the *Agrobacterium*-mediated transformation protocol for *C. vulgaris,* (2) the determination of the impact of *Cvudgat1* overexpression on the oil-productivity in *C. vulgaris*, and (3) the identification and characterization of CvuWRI1 for co-overexpression in *C. vulgaris* and investigation of its impact on TAG-biosynthesis.

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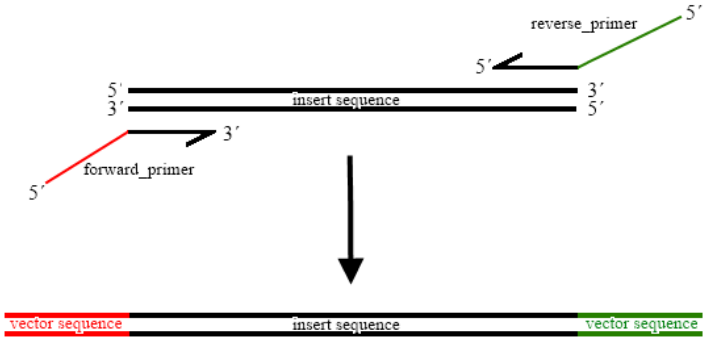
Appendix

3.I : Overlap-Extension PCR

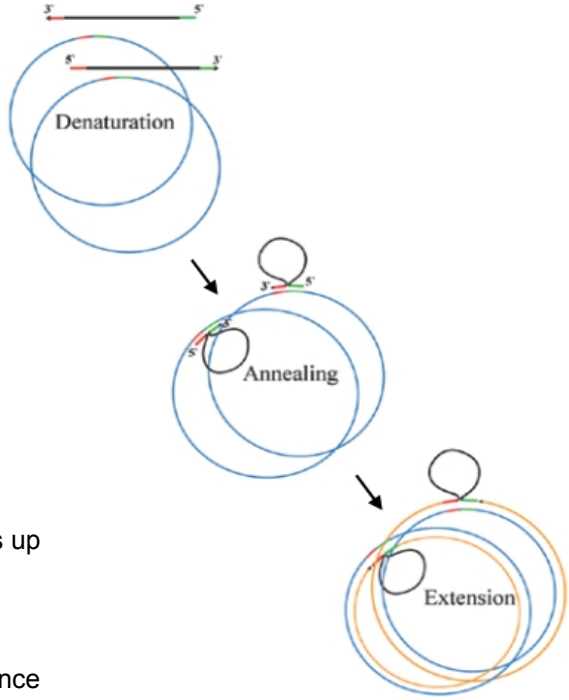
(A) Megaprimer PCR:

- the first of two PCRs
- creates an insert with vector sequences at both ends the product will be used as primers (megaprimers) for the OE-PCR



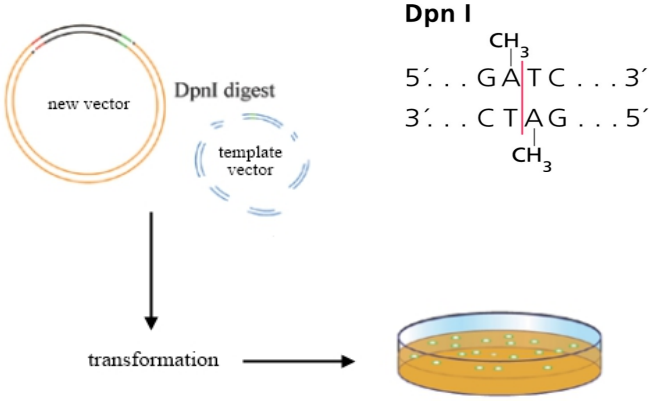
(B) OE-PCR:

- *approach:*
  → vector = template
  → primers = megaprimers
  → phusion® polymerase
  → most efficient with 17 – 18 cycles
- *PCR:*
  → *denaturation*: megaprimers & vector dsDNAs get separated
  → *annealing:* megaprimers binds to vector sequence on both strands
  → *extension:* the DNA-polymerase builds up the entire vector backbone resulting in the new vector containing the insert sequence



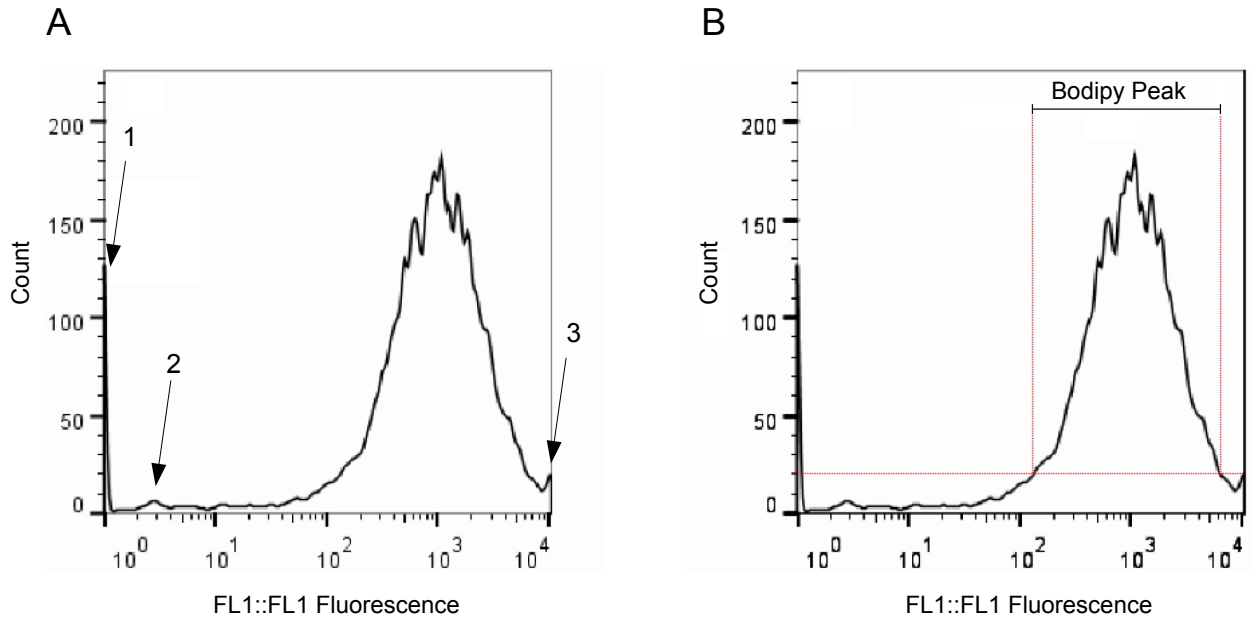
(C) DpnI digestion & transformation

- DpnI digestion:
  → digestion of methylated DNA
  → inactivation of endonuclease
  → re-hybridization of vectors
- transformation:
  → no purification step required
  → chemical or electro-competent *E. coli* strains can be used



3.II : Elimination of Background Fluorescence with FlowJo

The analysis and evaluation of the raw flow cytometry data were performed with the software FlowJo vX.0.7. With this, the histograms of the FL1 detector were altered for calculation of the fluorescence mean. To avoid background fluorescence (Figure A) in the calculated mean, values were excluded, when its fluorescence was counted less than 20-times (Figure B). The new fluorescence mean of the data set was calculated by the program automatically.



Histogram of the detected Bodipy signal by the FL1 detector. (A) The arrows show at signals with background fluorescence, including the fluorescence intensity of 1.0 (1), intensities with low background (2), or with signals higher than the scale. **(B)** The perimeter with signals counted more than 20-times and within the peak were used to determine the new and more accurate fluorescence mean.

3.III : Example Calculation for Flow Cytometry Data

Example data:

	FL1 fluorescence mean		
	Positive control	**Negative control**	**Sample**
1. measurement	850	265	652 ↑
2. measurement	870	284	589 ↓
3. measurement	865	244	611
Average	862	264	617

- Appoint positive control to 100 % and negative control to 0 %:

$$\frac{862 - 264}{100} = 5.98$$

→ That implies, a fluorescence intensity of 5.98 equals 1 % in respect to the positive (100 %) and negative (100 %) control.

- Sample calculation with respect to the controls:

$$\frac{617 - 264}{5.98} = 59\%$$

→ That implies, the intensity of the sample lead us to the conclusion that this yeast culture contains 59 % oil in respect to the positive and negative controls with 100 % and 0 % oil, respectively

- Deviation calculations with respect to the controls:

→ upper deviation:

$$\frac{652 - 264}{5.98} = 65\%$$

→ lower deviation:

$$\frac{589 - 264}{5.98} = 54\%$$

→ That implies, the upper and lower deviation of the sample with an average of 59 % is 65 % and 54 %, respectively, with respect to the positive (100 %) and negative (100 %) control.

That is how all the calculations were made to obtain the data for the representation of the “Impact of *Cvudgat1* expression in non-oil-producing yeast mutants” (Chapter 4, Section 4.3.4., Figure 4.7). Additionally for the calculation of these results, the data of four independent experiments with three biological replicates were combined, whereas the error bars indicate the fluctuations of the values between 12 replicates.

4.I : DGAT1 Highly Conserved Regions

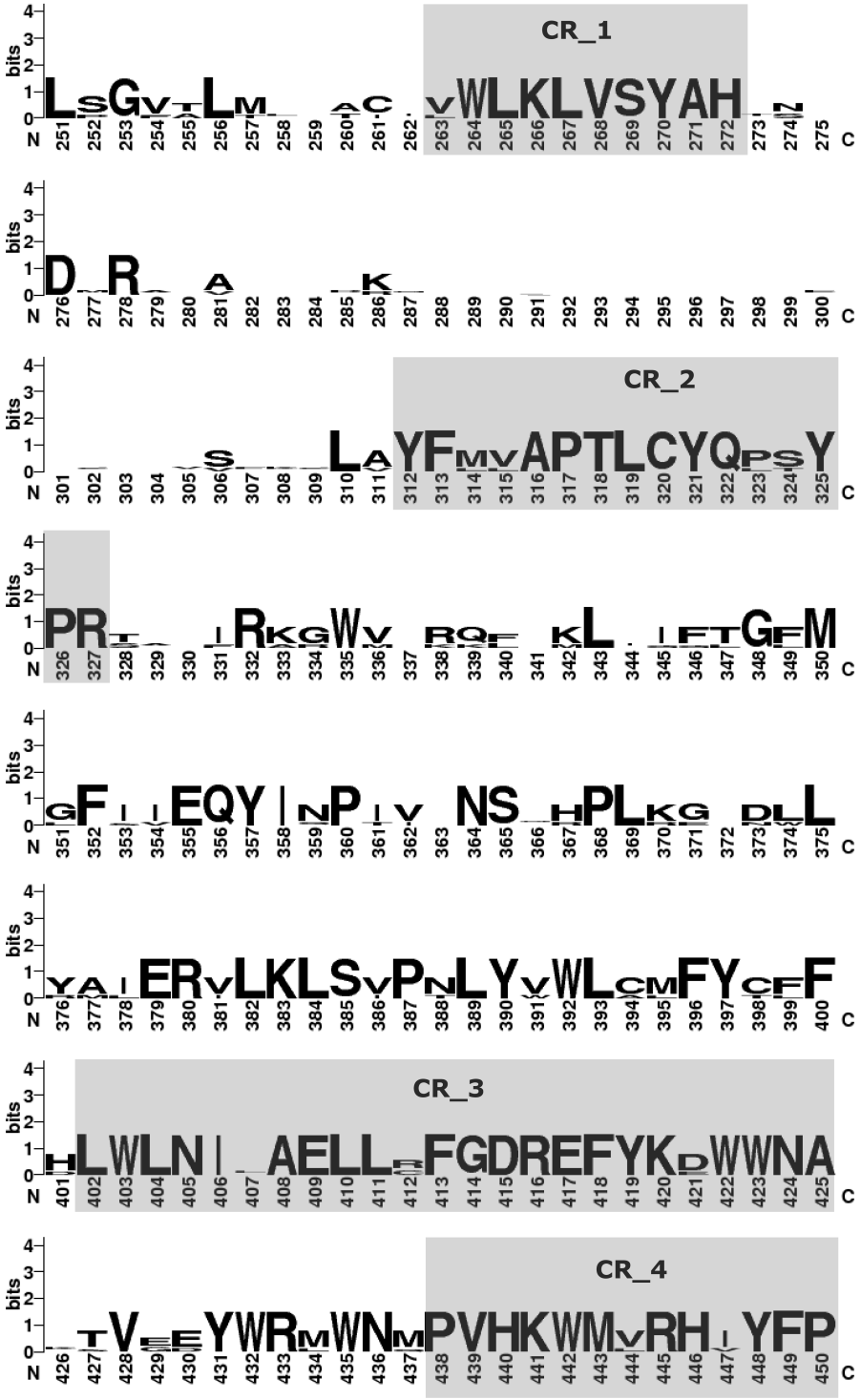


Figure S.I | Alignment of DGAT1 protein sequences from higher plants and a microalga to identify highly conserved regions (CR). Higher plants: *Arabidopsis thaliana* (AtDGAT1; NP179535.1), *Olea europaea* (OeDGAT1, AAS01606.1), *Vernicia fordii* (VfDGAT1, ABC94471.1), and *Ricinus communis* (RcDGAT1, XP002514132.1). Microalga: *Chlorella variabilis* (CvaDGAT1, EFN50697.1). The alignment was compiled by WebLogo 3 (Cooks *et al.* 2004).

Cooks, Gavin E.; Hon, Gary; Chandonia, John-Marc; Brenner, Steven E. (2004): WebLogo: A Sequence Logo Generator. In: *Genome Research* 14, pp. 1188-1190.

4.II : Primer Binding Regions for *Cvudgat1* Identification by Nested-PCR

>*Chlorella variabilis dgat1* cDNA sequence

```
ATGCCACACCACACGCACCCCACCTTAACCTCCCTCCTCTTCTCCCACCCTCCTCCTCCCCTCCCCTCTCTCCCACCCCACCCCCACCCA
  M  P  H  H  T  H  P  T  L  T  S  L  L  F  S  H  P  P  P  P  L  P  S  L  P  P  H  P  H  P

GGCGTCATCACCCTGGTCTTCCTCATCCTGGCCGCCACCAACTTCCGCCTCATCCTGGAGAACATGATCAAGTATAGGCTGAGGTTCAAC
  G  V  I  T  L  V  F  L  I  L  A  A  T  N  F  R  L  I  L  E  N  M  I  K  Y  R  L  R  F  N

CCGCTCACATTCCTGCGCACCGCGCTCACGCCCTCGGGCAACCTGCCGCTGCTGCTGTGCTGGCCGCTGCTGGCCTGCTTCGCGCTGTGC
  P  L  T  F  L  R  T  A  L  T  P  S  G  N  L  P  L  L  L  C  W  P  L  L  A  C  F  A  L  C

GCGCTGGGCATCGAGCGCTTTGCGCACCTGGTGTTCCTGCTCAACCTGCTCAACACCAGCGCCGCGCTGCTGGCCCCCTGCGCCATCATC
  A  L  G  I  E  R  F  A  H  L  V  F  L  L  N  L  L  N  T  S  A  A  L  L  A  P  C  A  I  I

                                                                                CvaCR1DGAT_f
CTGCACACCCGCGCCGAGCTGCTGCCCGGCTTTGCGCTCACCATGGCCACAATCGTGCTGTGGCTCAAGCTGGTGTCGTATGCGCACGTC   CR_1
  L  H  T  R  A  E  L  L  P  G  F  A  L  T  M  A  T  I  V  L  W  L  K  L  V  S  Y  A  H  V

AACTGGGACTACAGGTGGGCCGTGTGCCCCGGGGAGCGCGGCAGCGGGGTGGTGCCGGAGGGCGTGGAGGAGGAGCTGCGGTACCCCGAG
  N  W  D  Y  R  W  A  V  C  P  G  E  R  G  S  G  V  V  P  E  G  V  E  E  E  L  R  Y  P  E

                                                                  CvaCR2DGAT_f
AACATCGTGGCCGCCAACCTGGCATACTTCCTGCTGGCGCCCACGCTGTGCTACCAGCTCACCTACCCGCGCAGCAAGCGGTTCCGGGCA   CR_2
  N  I  V  A  A  N  L  A  Y  F  L  L  A  P  T  L  C  Y  Q  L  T  Y  P  R  S  K  R  F  R  A

CGGTGGATGGCCAAGAAGCTGCTGATGCTGACGGGGGGCCTGGGCCTGATGCTGTTCAGCATCGAGCAGTACATCCAGCCCACCATCGAC
  R  W  M  A  K  K  L  L  M  L  T  G  G  L  G  L  M  L  F  S  I  E  Q  Y  I  Q  P  T  I  D

AACAGCATGCGGCCGCTGAGGGAGATGGACTGGCTGCGCATGCTGGAGCGCATCCTCAAGCTGTCCATCCCCACCCTCTACTGGTGGCTG
  N  S  M  R  P  L  R  E  M  D  W  L  R  M  L  E  R  I  L  K  L  S  I  P  T  L  Y  W  W  L

                                                                                CvaCR3DGAT_r
GCCATGTTCTACACGCTCTTCGACCTGTGGCTCAACATCATCGCAGAGCTGCTGCGCTTCGGGGACCGCGAGTTCTACAAGGAGTGGTGG   CR_3
  A  M  F  Y  T  L  F  D  L  W  L  N  I  I  A  E  L  L  R  F  G  D  R  E  F  Y  K  E  W  W

                                                                  CvaCR4DGAT_r
AATGCCACCACCGTGGGCGAGTACTGGCGGCTGTGGAACCAGCCGGTGCACAAGTGGATGCTGCGACACGTCTACTTCCCGCTCATCCGC   CR_4
  N  A  T  T  V  G  E  Y  W  R  L  W  N  Q  P  V  H  K  W  M  L  R  H  V  Y  F  P  L  I  R

CACGGGGTGCCCAAGTTCCACGCAGGCCTCATGGTGTTCTTTGTGAGCGCCGTGTTCCACGAGGTGCTGGTGGGCGTGCCGCTGCACATG
  H  G  V  P  K  F  H  A  G  L  M  V  F  F  V  S  A  V  F  H  E  V  L  V  G  V  P  L  H  M

CTGCGGCTGTGGGCCTTTTGGGGCCTCATGATGCAGGTGCCGCTGATGATAGTGACGGAGTGGCTCAAGGGGCGGCTGCGGAGCGACCGC
  L  R  L  W  A  F  W  G  L  M  M  Q  V  P  L  M  I  V  T  E  W  L  K  G  R  L  R  S  D  R

GTGGGCAACATCATCTTCTGGGTGTCCTTCTGCTTTGTGGGGCAGCCGCTGGCCATGATCCTGTACTATCACGACTTCCGCCAGGTGCGC
  V  G  N  I  I  F  W  V  S  F  C  F  V  G  Q  P  L  A  M  I  L  Y  Y  H  D  F  R  Q  V  R

TGTTGCACAGCCGCAGCATGTGCAACATGTATTGTTGCGGTACAGGACGGCGTCGGATGGTGGATGCTGCCGGGTTGA
  C  C  T  A  A  A  C  A  T  C  I  V  A  V  Q  D  G  V  G  W  W  M  L  P  G  -
```

***Chlorella variabilis dgat1* cDNA and amino acid sequences for primer derivation.** The conserved regions (CR_1 to CR_4) are highlighted in light gray. Dark gray and underlined letters indicate the sequences of the primers CvaCR**1**DGAT_f, CvaCR**2**DGAT_f, CvaCR**3**DGAT_r, and CvaCR**4**DGAT_r (Chapter 3, Section 3.1.8.; Table 3.7; 5´- end of some primers were edited for primer optimization). The primers were used for the nested PCR to obtain parts of *C. vulgaris dgat1* cDNA sequence. *C. vulgaris* cDNA libraries served as template in the primary nested PCR.

4.III : *Cvudgat1* gDNA Sequence

>*Cvudgat1* gDNA sequence(2,854 bp)

0001 – ATGGCGCCCC GCGCAAGCCT GCTGTCTTCA GAGCGCATCC GCCTGCTCAA TCAGAGCGGT – 0060
0061 – GAGGCGAGCA GTTCGGCCAA ACTCTCCTTT GGTCTGCCGC TTTGGTTTGA ACCCCCGGTG – 0120
0121 – GGCTAGCCCC TAGCCCCTAG CCAACTGTCA TAGCGCTCAG CCCGCTTGGC AGCTCCACGC – 0180
0181 – TGCTGTGCCA CGCCTGTCTC TGCTGACCCT GCGGGGGCTG AGCTGCTTTC TCTCCTCCCC – 0240
0241 – CTTGCGCAGG TGTGGTGACG CTGGTGTTCA TCATCCTGGC GGCCGCCAAT TTCCGCCTCA – 0300
0301 – TCCTGGAGAA CATCATGAAG TATGGGCTGC GCTTCAACCC CTTCTCCTTC CTGAAAAACG – 0360
0361 – CCATCACACC TTCTGGTGCG TGTGCCCGTG GATGTGCGTG CTGTTGTGCT GATGTGTGTG – 0420
0421 – GAATCCATGC GTCTAGTTTG TGCCCCGAGG AAGGACTGGC TGCTCTGAGA AGGGAGCAAA – 0480
0481 – GTACAGACCT GCACATGACG CACTGTGGCA GCACAGCATG GAGCGGCCTG TGTGTCTGCC – 0540
0541 – ATCCCCTGTG TTGGAGCAGG TTGCGTGCAT GTTCATGCCT GGCGCGTGAA TGTCTGCATG – 0600
0601 – CTGCGATCGC AACCTGGTTC CGCCTGCAGG CAACACGCCG CTGGTGCTGT GCTGGCCGCT – 0660
0661 – GCTGGCCTTC TTTGCGCTGA GCGCCCTGGC CATCGAACGG TTTGCTGTCA GGCTGCTGGA – 0720
0721 – CATGGAGCGG CGTGCGGCAG CTGTCACCGA CAAGCGCTCG GTTGGGTACA CTGAGATGAA – 0780
0781 – CAAGGCCGCA GCTCGACGCG CCAGCGCAAC TGGTGAGCTG CTGGCAACCC TGCAAGCGAT – 0840
0841 – TGTGCACATC GTGGCACAAC ATGCCCTGTG CTGATGTGTT GGTCGTGACT TTCTGTTCAT – 0900
0901 – CATGTCATCT CCTCCCCTGC TGCCTGCAGA GCATCTGGTG TTTGTGCTCA ACCTGCTCAA – 0960
0961 – CACCACTGCC GCGCTGCTTG CCCCCTGCGC CATCATCCTG CACACCTGCG CTGAGCTGAT – 1020
1021 – GCCTGGCTTT GCCCTCACCA TGGCCACGTG CGTGCTCTGG CTCAAGCTGG TCAGCTATGC – 1080
1081 – CCATGTGAAC TTTGATTATC GGTGAGTCTG CATGACAAGC GTTGGGCCCA TGTCGCTGTC – 1140
1141 – GGCTTGCAAT GCTGCGATGT GCACCATGGG AGGCCGATAT GTGGCGCTGC TTTCGATCGT – 1200
1201 – TTGCTTTGTT GCCTGTGTGT GCTCAACAAT GCCTGCTGCC CTACCGAGCA ATGACTGGCA – 1260
1261 – CAGAGCACTG ATTGGCACTC CTCCTTGCTC CCCTGGACGC TCTTCTGCAC CGCAGTGTGG – 1320
1321 – CGAGGCGGCG CGGCGAGACA CGCCCCGGGG AGCGTGGCAG TGGGCAGTAC CCTGAGAACG – 1380
1381 – TGGAGCAGGA GCTTCGCTAT CCTGAAAACA TTGCTCTGGG CAACCTGGCG TACTTCCTGG – 1440
1441 – CAGCGCCCAC GCTGTGTTAC CAGATCACGT ACCCTCGCAG GTACGCCAGC TGGCTGTGGG – 1500
1501 – GGTTGCGCTG GCGCTGTCCG GTGTGAGCAA GCGTGACCCC ACAGCTCTGG CATCTGTGTC – 1560
1561 – GCTTTTGGGA CTAATGGCTT TGCTGGTTGT CGCTGCTACG AAACGCCTTG GCCTGGACCA – 1620
1621 – GCTGTGGTCA GTCAAGCACA TGAATTGCTG CCCTGGCGTA CAACTCCTGT ACCCTGTGCT – 1680
1681 – CCCTGCTTCT GCTCCTTGTG TGTGCAGCAA GCGGTTCCGT GCCCGCTGGA TGGTCAAGAA – 1740
1741 – GCTGTTCATG TTCACTGGAG GGCTGGGCCT GATGCTGTTT GTGACGGAAC AGTACATTCA – 1800
1801 – GCCCACCATC GACAACAGCA TGCGGCCGCT GAAGGAGATG GTGCGGAACT GCTGCAATCG – 1860
1861 – ATGGCTTCTC ATGCGCCTGG CGCCTGGTGT ATTGCTGTTC CAGCGTCCCT AGCCCTCAGC – 1920
1921 – CATCCACTCT TGACATCAAT GTTGATGTCG CGTGTTGGTT TACCCTCCCC AAACCGCTTG – 1980
1981 – CCTGCCTGCC TGCCTGCAGG ACTGGCTGCG CATGCTGGAG CGCATCCTGA AGCTGTCCAT – 2040
2041 – CCCCACTCTC TACTGGTGGC TGGCCATGTT CTACACCCTA GGAGTGGTGG AATGCGACTA – 2100
2101 – CTGTTGGCGA GTACTGGCGC NTCTGGAACC AGCCCGTGCA CAAGTGGATG CTGCGCCACG – 2160
2161 – TGTACTTCCC CNTCNTGCGC CACAAAGTGC CAAAGTTCTA TGCTGGTGAG CAGGCGGTGG – 2220
2221 – CGTGTTCTTG TGCATGGTGT GAAGTTGGCC GACGTGTCAT GAAGACGGCG CGGGATGATG – 2280
2281 – AGGCAGCGGT CCATGATCTA CTCACAATAA AACTATCCTG AATGATATAG ACACTATTCC – 2340
2341 – AACGTTCTTG CCGTGGCGTT CAATTACACT CCCCCTCTCC TTGCAGGCTT TGCTGTGTTT – 2400
2401 – TTCATTTCCG CTGTATTCCA CGAGGTGTTG GTGGGCGTGC CGCTGCACAT GGTGCGGCTG – 2460

```
2461 – TGGGCCTTCT GGGGCCTGAT GGCGCAGGTG GGTGGGTGGG TGTGCCGCGG CGGCAGGGAG - 2520
2521 – TGCAACTGCA CGACCTCAGC ATTTAGTGTT TTGCGTGACT GGTCACACTG TGAGGCTTCT - 2580
2581 – TTCTGAACAC CACAACAAGA TCCACCCGAA CAAGATCAAG TTCGCTTCCA TGAAGACGAC - 2640
2641 – AATTTGCCCT TGTCCCTGCT GTCTTGTCTC CTGGCAGGTG CCGCTTATGA TCGTGACTGA - 2700
2701 – GTGGCTGAAG AACAAGCTGA AGAACGATCG CATTGGCAAC ATCGTGTTCT GGATATCGTT - 2760
2761 – CTGCTTCGTG GGGCAGCCGC TGGCCATGAC ACTCTACTAC CATGACCATC TGCAGGGCTA - 2820
2821 – CAAGCACCCC TTCCAGGGCA CGCCCATTAT CTGA                              - 2854
```

The gray highlighted sequence parts label exons, the not marked regions indicate sequence areas located in an intron. The complete cDNA sequence is shown in the following Supplementary Material 4.III.

4.IV : *Cvudgat1* cDNA Sequence

>*Cvudgat1* cDNA sequence(1,383bp)

```
0001 - ATGGCGCCCC GCGCAAGCCT GCTGTCTTCA GAGCGCATCC GCCTGCTCAA TCAGAGCGGT – 0060
0061 - GTGGTGACGC TGGTGTTCAT CATCCTGGCG GCCGCCAATT TCCGCCTCAT CCTGGAGAAC – 0120
0121 - ATCATGAAGT ATGGGCTGCG CTTCAACCCC TTCTCCTTCC TGAAAAACGC CATCACACCT – 0180
0181 - TCTGGCAACA CGCCGCTGGT GCTGTGCTGG CCGCTGCTGG CCTTCTTTGC GCTGAGCGCC – 0240
0241 - CTGGCCATCG AACGGTTTGC TGTCAGGCTG CTGGACATGG AGCGGCGTGC GGCAGCTGTC – 0300
0301 - ACCGACAAGC GCTCGGTTGG GTACACTGAG ATGAACAAGG CCGCAGCTCG ACGCGCCAGC – 0360
0361 - GCAACTGAGC ATCTGGTGTT TGTGCTCAAC CTGCTCAACA CCACTGCCGC GCTGCTTGCC - 0420
0421 - CCCTGCGCCA TCATCCTGCA CACCTGCGCT GAGCTGATGC CTGGCTTTGC CCTCACCATG - 0480
0481 - GCCACGTGCG TGCTCTGGCT CAAGCTGGTC AGCTATGCCC ATGTGAACTT TGATTATCGT - 0540
0541 - GTGGCGAGGC GGCGCGGCGA GACACGCCCC GGGGAGCGTG GCAGTGGGCA GTACCCTGAG - 0600
0601 - AACGTGGAGC AGGAGCTTCG CTATCCTGAA AACATTGCTC TGGGCAACCT GGCGTACTTC - 0660
0661 - CTGGCAGCGC CCACGCTGTG TTACCAGATC ACGTACCCTC GCAGCAAGCG GTTCCGTGCC - 0720
0721 - CGCTGGATGG TCAAGAAGCT GTTCATGTTC ACTGGAGGGC TGGGCCTGAT GCTGTTTGTG - 0780
0781 - ACGGAACAGT ACATTCAGCC CACCATCGAC AACAGCATGC GGCCGCTGAA GGAGATGGAC - 0840
0841 - TGGCTGCGCA TGCTGGAGCG CATCCTGAAG CTGTCCATCC CCACTCTCTA CTGGTGGCTG - 0900
0901 - GCCATGTTCT ACACCCTCTT TGACCTGTGG CTCAACATCC TGGCAGAAGT GCTGCGATTT - 0960
0961 - GGCGACCGGG AATTCTACAA GGAGTGGTGG AATGCGACTA CTGTTGGCGA GTACTGGCGC - 1020
1021 - CTCTGGAACC AGCCCGTGCA CAAGTGGATG CTGCGCCACG TGTACTTCCC CCTCCTGCGC - 1080
1081 - CACAAAGTGC CAAAGTTCTA TGCTGGCTTT GCTGTGTTTT TCATTTCCGC TGTATTCCAC - 1140
1141 - GAGGTGTTGG TGGGCGTGCC GCTGCACATG GTGCGGCTGT GGGCCTTCTG GGGCCTGATG - 1200
1201 - GCGCAGGTGC CGCTTATGAT CGTGACTGAG TGGCTGAAGA ACAAGCTGAA GAACGATCGC - 1260
1261 - ATTGGCAACA TCGTGTTCTG GATATCGTTC TGCTTCGTGG GGCAGCCGCT GGCCATGACA - 1320
1321 - CTCTACTACC ATGACCATCT GCAGGGCTAC AAGCACCCCT TCCAGGGCAC GCCCATTATC - 1380
1381 - TGA                                                              - 1383
```

4.V : CvuDGAT1 Protein Sequence

>CvuDGAT1 protein sequence(460 aa)

001 - MAPRASLLSS ERIRLLNQSG VVTLVFIILA AANFRLILEN IMKYGLRFNP FSFLKNAITP – 060

061 - SGNTPLVLCW PLLAFFALSA LAIERFAVRL LDMERRAAAV TDKRSVGYTE MNKAAARRAS – 120

121 - ATEHLVFVLN LLNTTAALLA PCAIILHTCA ELMPGFALTM ATCVLWLKLV SYAHVNFDYR – 180

181 - VARRRGETRP GERGSGQYPE NVEQELRYPE NIALGNLAYF LAAPTLCYQI TYPRSKRFRA – 240

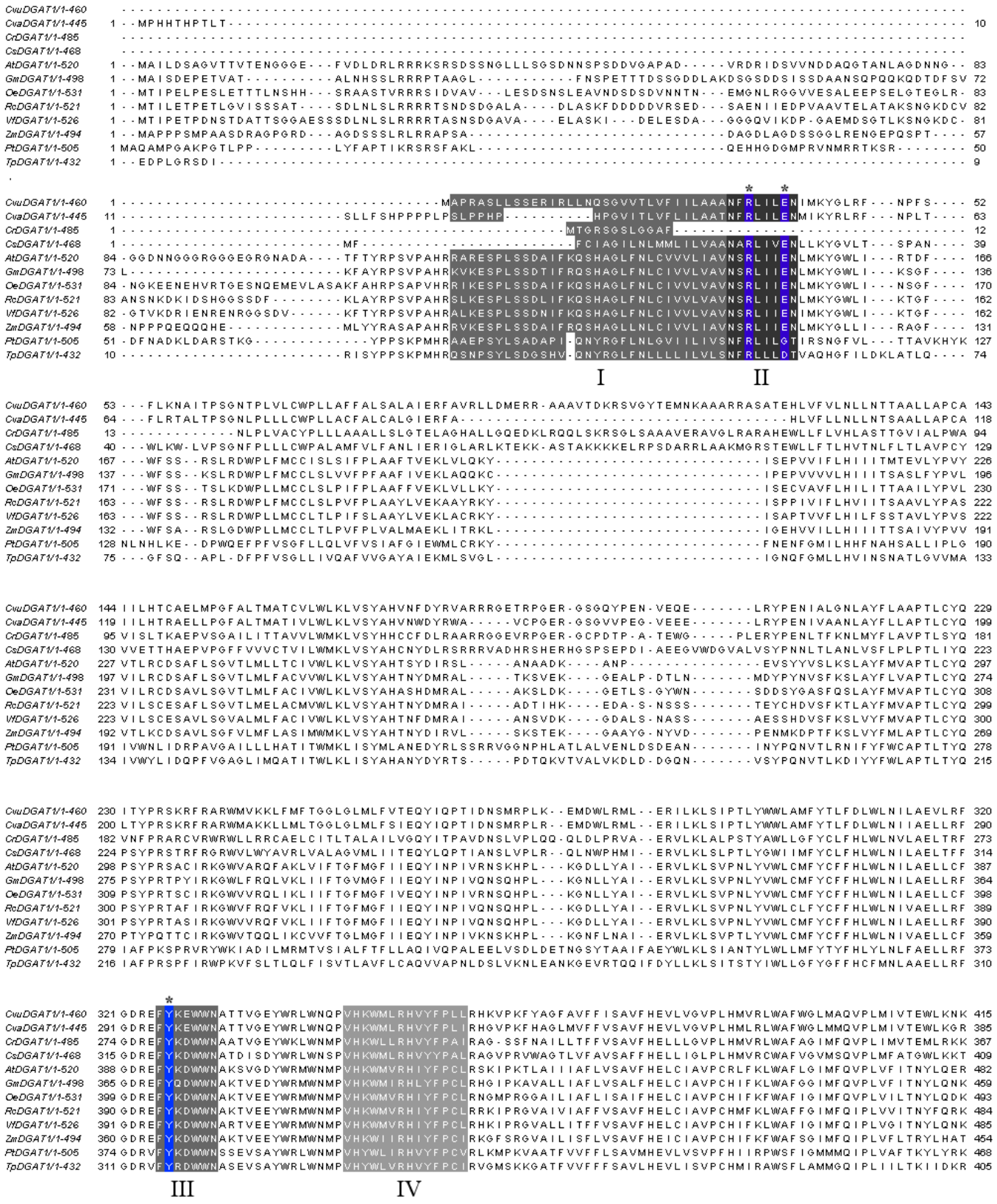
241 - RWMVKKLFMF TGGLGLMLFV TEQYIQPTID NSMRPLKEMD WLRMLERILK LSIPTLYWWL – 300

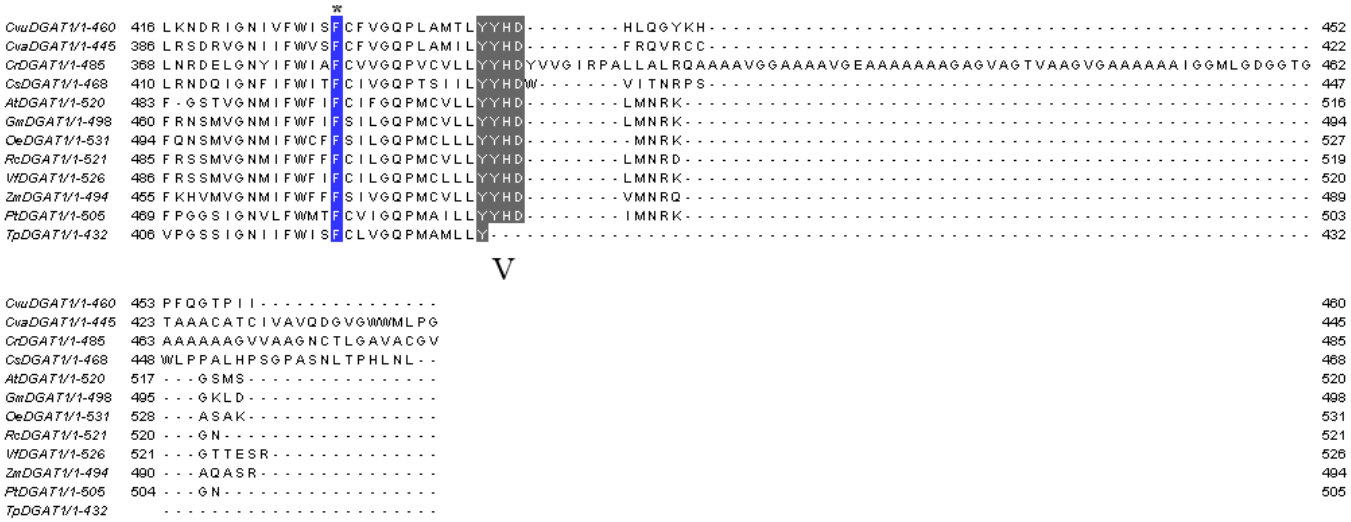
301 - AMFYTLFDLW LNILAEVLRF GDREFYKEWW NATTVGEYWR LWNQPVHKWM LRHVYFPLLR – 360

361 - HKVPKFYAGF AVFFISAVFH EVLVGVPLHM VRLWAFWGLM AQVPLMIVTE WLKNKLKNDR – 420

421 - IGNIVFWISF CFVGQPLAMT LYYHDHLQGY KHPFQGTPII - - 460

4.VI : Motif Identification in CvuDGAT1 – Complete Alignment





Sequence homology comparison of CvuDGAT1 with DGAT1 protein sequences from green algae, diatoms, and higher plants. The CvuDGAT1 sequence was aligned against characterized DGAT1 amino acid sequences from green algae, plants, and diatoms with MEGA 6 (Tamura *et al.* 2013). **Green algae**: *Chlorella variabilis* (CvaDGAT1, EFN50697.1), *Chlamydomonas reinhardtii* (CrDGAT1, (Boyle *et al.* 2012) , and *Coccomyxa subellipsoidea* (CsDGAT1, EIE20990.1); Oil-producing or model **plants**: *Arabidopsis thaliana* (AtDGAT1; NP179535.1), *Glycine max* (GmDGAT1, AAS78662.1), *Olea europaea* (OeDGAT1, AAS01606.1), *Ricinus communis* (RcDGAT1, XP002514132.1), *Vernicia fordii* (VfDGAT1, ABC94471.1), *Zea mays* (ZmDGAT1, ABV91586.1); **diatoms**: *Phaeodactylum tricornutum* (PtDGAT1, ADY76581.1) and *Thalassiosira pseudonana* CCMP1335 (TpDGAT1, XP_002287215.1). Conserved motifs and putative signatures are numbered from I to V (see text for details). We identified the acyl-CoA binding motif (**I**), the putative active site of DGAT1 (**II**, black box), the highly conserved fatty acid protein signature (**III**), containing a putative tyrosine phosphorylation site (●), theChlamydomonas *reinhardtii* DAG/phorbol ester binding motif (**IV**), and the putative retrieval motif (**V**). Within the predicted active site (II) the amino acids arginine (R; denoted by asterisk) and glutamine (E; denoted by asterisk) play important roles in the enzymatic reaction. The highly conserved amino acid phenylalanine (F) is highlighted in white with black background. One sequence part were cut out. Its position is marked with a vertical line.

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Curriculum Vitae

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Publications

expected in 2015	Publication of **Chapter 4** with minor edits & additional data Preliminary title: „*Identification and Characterization of Diacylglycerol Acyltransferase Type-1 (DGAT1) from Chlorella vulgaris*“
29. - 30.07.2014	Biomass 2014: *Growing the Future Bioeconomy Bioproducts*, Washington D.C. (Washington, USA) Poster: „*Identification and Characterization of Diacylglycerol Acyltransferase Type-1 (DGAT1) from Chlorella vulgaris*“
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29. - 30.03.2014	78th Annual Meeting of the Northeast Section of the American Society of Plant Biologists (NEASPB), Kingston (Rhode Island, USA) Talk: *„Identification and Characterization of Diacylglycerol Acyltransferase Type-1 (DGAT1) from Chlorella vulgaris*“