The production of VLCPUFAs in plants

PhD Thesis

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Affidavit

Herewith I affirm that I wrote this PhD Thesis independently and with no other sources and aids than quoted.

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List of Publications

Journal article

Wagner M, **Hoppe K,** Czabany T, Heilmann M, Daum G, Feussner I, Fulda M (2010) Identification and characterization of an acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) gene from the microalga *O. tauri*. Plant Physiology and Biochemistry **48:** 407-416

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Abstract

Very long-chain polyunsaturated fatty acids (VLCPUFAs) like eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are health-beneficial components in the human diet. However, due to overfishing and pollution of the sea, oily sea fish as the main dietary source for these important fatty acids is decreasingly available. A promising alternative to this shortfall is the production of VLCPUFAs in transgenic oilseed crops. Although this goal has already been reached to some extent, there are still challenges which need to be met. These are the specific accumulation of VLCPUFAs in the neutral lipid fraction of seeds as well as absolute product yields, especially in case of DHA. Those aspects of heterologous VLCPUFA production might be improved by transfer of specific acyltransferases and superior desaturases from suitable VLCPUFA-producing organisms into seed lipid metabolism. Therefore, nucleotide sequences from two *Ostreococcus* species potentially encoding acyltransferases or a Δ4-desaturase, respectively, were characterized by expression in yeast. In these studies, none of the analyzed putative acyltransferases revealed specificity for VLCPUFAs. In contrast, the front-end desaturase Old4p from *Ostreococcus lucimarinus* was found to prefer VLCPUFAs to shorter-chain fatty acids and to desaturate both (n-3)- and (n-6)-substrates bound to lipids. In addition to the yeast experiments, acyltransferases were also introduced individually and in combination with VLCPUFA-producing enzymes into *Arabidopsis thaliana*. Upon separate expression, a putative acyl-coenzyme A:lysophosphatidylcholine acyltransferase (LPCAT) sequence from *O. lucimarinus* produced the strongest effects by enhancing levels of polyunsaturated fatty acids at the expense of monounsaturated fatty acids in total seed lipids. However, the combination of acyltransferases with VLCPUFA-producing enzymes did not lead to higher yields in transgenic seeds. Besides the expression studies performed with the microalgal acyltransferase sequences, also endogenous acyltransferase activities of the two different host plants *A. thaliana* and *Camelina sativa* were investigated by using both species for VLCPUFA-production and comparing product yields.

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Abbreviations

ACP	acyl carrier protein
ad	up to
ALA	α-linolenic acid (18:3(n-3))
ALE1	*Saccharomyces cerevisiae* acyltransferase for lyso-phosphatidylethanolamine
ARA	arachidonic acid (20:4(n-6))
ATP	adenosine 5'-triphosphate
bp	base pair(s)
CDP	cytidine 5'-diphosphate
CL	cardiolipin
CoA	coenzyme A
CPT	CDP-choline:diacylglycerol cholinephosphotransferase
CsMGDGS	*Cucumis sativus* monogalactosyldiacylglycerol synthase
Da	Dalton
daf	days after flowering
DAG	diacylglycerol
dap	days after pollination
ddH_2O	double distilled water
DDT	DAG-DAG transacylase
DGA1	*Saccharomyces cerevisiae* acyl-CoA:diacylglycerol acyltransferase
DGAT	acyl-CoA:diacylglycerol acyltransferase
DGDG	digalactosyldiacylglycerol
DHA	docosahexaenoic acid (22:6(n-3))
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide
DPA	docosapentaenoic acid (22:5(n-3))
Egd4	*Euglena gracilis* Δ4-desaturase
EPA	eicosapentaenoic acid (20:5(n-3))
ER	endoplasmic reticulum
et al.	et alii, et aliae; and others
FAD	fatty acid desaturase

FAME	fatty acid methyl ester
FAR1	fatty acid reductase 1
FAS	fatty acid synthase
FID	flame ionization detector
g	gramm
G3P	glycerol-3-phosphate
GC	gas chromatography
GPAT	glycerol-3-phosphate acyltransferase
HPLC	high-performance liquid chromatography
l	liter
LA	linoleic acid (18:2(n-6))
LPAAT	lysophosphatidic acid acyltransferase
LPCAT	acyl-Co:lyso-phosphatidylcholine acyltransferase
LPLAT	acyl-Co:lyso-phospholipid acyltransferase
LRO1	*Saccharomyces cerevisiae* LCAT-related protein 1
MAG	monoacylglycerol
MGAT	acyl-CoA:monoacylglycerol acyltransferase
MGDG	monogalactosyldiacylglycerol
min	minute(s)
Ms3	Msd6-PSE1-Msd5-pCAMBIA3300
Msd5	*Mantoniella squamata* Δ5-desaturase
Msd6	*Mantoniella squamata* Δ6-desaturase
OD_x	optical density at x nm
Old4	*Ostreococcus lucimarinus* Δ4-desaturase
OlLPCAT	*Ostreococcus lucimarinus* acyl-Co:lysophosphatidylcholine acyltransferase
Ot3	Otd6-PSE1-Otd5-pCAMBIA3300
Otd5	*Ostreococcus tauri* Δ5-desaturase
Otd6	*Ostreococcus tauri* Δ6-desaturase
OtDGAT	*Ostreococcus tauri* acyl-CoA:diacylglycerol acyltransferase
OtLPCAT	*Ostreococcus tauri* acyl-Co:lyso-phosphatidylcholine acyltransferase
OtPDAT	*Ostreococcus tauri* phospholipid:diacylglycerol acyltransferase
p	protein
PA	phosphatidic acid

PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PDAT	phospholipid:diacylglycerol acyltransferase
PDCT	phosphatidylcholine:diacylglycerol cholinephosphotransferase
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
pH	approximates to the negative decadal logarithm of the molar concentration of dissolved hydronium ions
PI	phosphatidylinositol
PKS	polyketide synthase
PS	phosphatidylserine
PSE1	*Physcomitrella patens* Δ6-elongase cDNA sequence
Pt3	Ptd6-PSE1-Ptd5-pCAMBIA3300
Ptd5	*Phaeodactylum tricornutum* Δ5-desaturase
Ptd6	*Phaeodactylum tricornutum* Δ6-desaturase
RNA	ribonucleic acid
s	seconds
SD	standard deviation
sn	stereospecific numbering
SQD	sulfoquinovosyldiacylglycerol
TAG	triacylglycerol
TLC	thin layer chromatography
TpLACS	*Thalassiosira pseudonana* long-chain acyl-coenzyme A synthetase
v/v	volume/volume
VLCPUFAs	very long-chain polyunsaturated fatty acids
w/v	weight/volume
WS	wax ester synthase
x g	relative centrifugal force; g-force

1 Introduction

Very long-chain polyunsaturated fatty acids (VLCPUFAs) contain 20-22 carbon atoms and have more than three double bonds. They are essential as structural components for membranes, for example in the human brain (Glomset, 2006), or as precursors for various cellular messengers (Jump, 2002). Especially due to this latter function, VLCPUFAs of the (n-3)-series are known to have health-beneficial effects like protection of the cardiovascular system (Simopoulos, 2002). VLCPUFAs are mainly produced by marine microalgae, which form the base of the aquatic food chain. Therefore, oily sea fish is rich in these fatty acids and currently constitutes the main direct nutritional source for humans, who themselves cannot produce VLCPUFAs *de novo*. Due to a worldwide decline of fish stocks and increasing pollution of the sea, however, oily sea fish alone is no longer able to cover the global demand for these valuable dietary components (Naylor et al., 2000; Hites et al., 2004). As a possible alternative to this shortfall, transgenic annual oilseed plants producing VLCPUFAs in their seed oil have emerged. In the past years, major advancements have been made towards VLCPUFA production in plants. Nevertheless, some hurdles still need to be overcome to reach satisfying levels of these fatty acids in oilseeds. Several different enzymatic activities for the primary biosynthesis of VLCPUFAs need to be transferred from an adequate gene donor into plants and expressed in a coordinated way in their seeds. Because the products of this primary biosynthesis should end up in the plant seed oil, they need to be incorporated into triacylglycerol (TAG) molecules. This in most of the cases cannot be successfully accomplished by the endogenous plant enzymes, which limits accumulation *in planta*. The distribution of VLCPUFAs within the different lipid pools of the plant cell thus is a problem, which might be solved by the transfer of acyltransferase activities from organisms producing VLCPUFAs into plants in addition to the primary biosynthetic activities.

The presented thesis deals with the characterization of different microalgal enzymatic activities considered to be able to establish VLCPUFA biosynthesis or to influence VLCPUFA distribution upon transfer into plants. The following introduction gives a brief overview about fatty acid and glycerolipid biosynthesis in plants, which are reviewed in more detail for example by (Ohlrogge and Browse, 1995; Harwood, 1996). Different types of acyltransferases and their role in the process of TAG formation will be presented. Also, the hitherto existing usage of acyltransferases in biotechnological engineering of plants will be explained. Relevance and biosynthetic pathways of VLCPUFAs as well as enzymes involved therein will be covered in the course of this introduction. And last but not least, the state of the art of VLCPUFA synthesis will be described.

1.1 Fatty acids and glycerolipids

Lipids are organic molecules, which are present in every living cell. The class comprises a wide range of substances, which can be fatty acid derivatives like glycerolipids or are, like pigments and secondary compounds, synthesized via pathways independent from fatty acid metabolism. Lipids serve lots of important functions in the cell. Glycerolipids, for example, act as signalling and energy storage molecules or as major components of biological membranes. These function, due to the insolubility of lipid molecules in water, as selective barriers between different compartments and enable a directed exchange of metabolites and energy. Membranes primarily consist of lipid bilayers and therein embedded proteins, their characteristics are thus mainly influenced by the composition of the incorporated lipid species (Buchanan et al., 2000; Berg et al., 2004).

1.1.1 The molecular structure of fatty acids

Fatty acids are the building blocks of complex lipids. They are composed of a highly reduced hydrocarbon chain of varying length and a terminal carboxyl group. They contain an even number of carbon atoms, which can vary between eight and 32. Fatty acids with 16 or 18 carbon atoms are most common in biological systems. Fatty acids can be either saturated or unsaturated. In the latter case, they feature one or several double bond(s) in *cis*-configuration between the carbon atoms at distinct positions. A prominent example for a monounsaturated fatty acid is oleic acid (18:1(n-9)) possessing 18 carbon atoms and one double bond at the ninth carbon atom proximal to the methyl end of the molecule. Another way to name this fatty acid is $18{:}1^{\Delta 9}$. The underlying Δ-nomenclature is indicating the distance between the double bond position and the carboxyl end of the molecule. In case of two or more double bonds in one fatty acid, double bonds are interrupted in nearly all cases by at least one methylene group. Decreasing length and increasing degree of unsaturation of a fatty acid are lowering the melting point of this fatty acid and its derivatives (Buchanan et al., 2000; Berg et al., 2004).

1.1.2 *De novo* biosynthesis of fatty acids

In plants, fatty acids are synthesized in the plastids by action of the fatty acid synthase (FAS) complex. This complex consists of several different proteins that altogether catalyze the formation of fatty acids from acetyl-coenzyme A (CoA) and malonyl-CoA. Fatty acid biosynthesis starts with the formation of malonyl-CoA by adenosine 5'-triphosphate (ATP)-dependent carboxylation of acetyl-CoA, catalyzed by the enzyme acetyl-CoA carboxylase. The malonyl moiety is transferred onto acyl carrier protein (ACP), the first component of the FAS complex, and thereupon decarboxylated. In the following condensation reaction, acetyl-CoA is added and three subsequent reactions

of reduction, dehydration and another reduction result in the formation of acyl-ACP. This acyl-ACP is then further elongated in two-carbon chain units by cyclic repetition of condensation with malonyl-CoA, two reduction steps and a dehydration reaction. The process ends as soon as 16:0-ACP or 18:0-ACP are formed (Ohlrogge and Jaworski, 1997). Some intermediates of fatty acid biosynthesis are barely detectable. From this fact it can be deduced that the interplay between the different enzymatic activities is highly efficient and some kind of substrate channeling occurs during fatty acid synthesis (Buchanan et al., 2000). 16:0-ACP and 18:0-ACP can be used as substrate for desaturation by ACP-dependent desaturases. These soluble enzymes mostly introduce double bonds at the Δ9-position. One prominent example for this type of enzyme is the stearoyl-ACP desaturase, which catalyzes the formation of oleoyl-ACP (Shanklin et al., 1991; Thompson et al., 1991). The acyl chains can also be directly transferred from ACP onto glycerolipids by distinct acyltransferase activities for the synthesis of “prokaryotic” lipids (Harwood, 1996). Alternatively, the acyl moiety of the acyl-ACP can be cleaved off by thioesterases and is exported to the cytoplasm. There it is, esterified to CoA, used as substrate for the synthesis of “eukaryotic” lipids at the endoplasmic reticulum (ER) (Roughan and Slack, 1982; Somerville and Browse, 1991). As fatty acids mostly do not exist as free molecules in the cell, but rather are bound to glycerolipids, glycerolipid composition and biosynthesis will be explained in the following sections.

1.1.3 The composition of glycerolipids

Glycerolipids are important for cell membrane structure as well as energy storage and take an active part in various signalling events. They consist of a glycerol backbone connected with up to three fatty acids via ester bonds. The positions of the fatty acids at the glycerol part of the molecule are given according to the stereospecific numbering (*sn*) nomenclature as *sn*-1, *sn*-2 or *sn*-3.

Glycerolipids can be subdivided into different classes. Glycolipids constitute one class and contain a sugar headgroup bound to glycerol instead of the third fatty acid. Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) or sulfoquinovosyldiacylglycerol (SQD) are members of this group and are predominantly found in plastidial membranes. Phospholipids, as the second class, serve important functions in membranes and in cell signalling. They exhibit a polar phosphate-containing headgroup connected to glycerol. The headgroup can consist either of phosphate alone, the derived lipid is then named phosphatidic acid (PA), or of phosphate connected with alcohols like glycerol, choline, ethanolamine, inositol or serine. The corresponding lipids are named phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) or phosphatidylserine (PS). Another representative of this group of lipids is the mitochondrial lipid cardiolipin (CL), which is composed of two PG molecules that are interconnected at their glycerol portion. Neutral lipids make up the third class of glycerolipids and do not feature a polar headgroup. Common members are monoacylglycerols (MAGs), diacylglycerols (DAGs) and triacylglycerols (TAGs). They contain one, two or three fatty acids, respectively,

esterified to the glycerol backbone. DAG acts as central precursor in lipid metabolism and as signalling molecule, whereas TAG is an essential storage compound in the cell (Buchanan et al., 2000; Berg et al., 2004).

1.1.4 Biosynthesis of glyco- and phospholipids

Plant membrane lipids can be formed in two different pathways. The “prokaryotic” pathway (Roughan and Slack, 1982; Somerville and Browse, 1991) initially leads to the formation of PA in plastids by sequential activity of acyl-ACP:glycerol-3-phosphate acyltransferase (GPAT) and acyl-ACP:lyso-phosphatidic acid acyltransferase (LPAAT) on glycerol-3-phosphate (G3P). PA can then be further converted into cytidine 5’-diphosphate (CDP)-DAG or DAG. These compounds are then used as substrates for formation of PG or galactolipids, respectively (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). Due to the distinct enzyme specificities of the participating acyl-transferases, lipids derived from this pathway are enriched in palmitic acid (16:0) at the *sn*-2 position and in most cases oleic acid at the *sn*-1 position. Fatty acids associated with complex lipids can be further modified by plastidial desaturation activities (Somerville and Browse, 1991).

The “eukaryotic” pathway basically employs the same acyltransferase activities and also leads at first to the formation of PA and subsequently of CDP-DAG or DAG. But in contrast to the “prokaryotic pathway”, the reaction series takes place at the ER and requires acyl-CoAs as substrates. DAG is further converted to PC or PE, CDP-DAG is used for formation of PG, PS or PI. Lipids derived from this pathway are enriched in 18-carbon chain fatty acids at the *sn*-2 position and palmitic acid at the *sn*-1 position (Ohlrogge and Browse, 1995). Fatty acids bound to PC derived from the “eukaryotic pathway” can be further modified by desaturases which convert oleic acid further into linoleic acid (18:2(n-6), LA) and α-linolenic acid (18:3(n-3), ALA) (Arondel et al., 1992; Okuley et al., 1994).

Both “prokaryotic” and “eukaryotic” pathways do not exist separated from each other, but they are interconnected by a common DAG pool (Browse et al., 1986). Also, lyso-PC and PA are transported between plastid and ER and are used for lipid synthesis at both sites (Roughan and Slack, 1982; Somerville and Browse, 1991). Thus, all glycerolipids in the cell are involved in continuous exchange. Furthermore, phospho-lipids underlie constant turnover at their *sn*-2 acyl moiety in the so-called Land’s cycle (Lands, 1958) by concerted action of phospholipases, acyl-CoA synthetases and lyso-phospholipid acyltransferases (LPLATs) (Fulda et al., 1997; Lands, 2000; Shimizu et al., 2006).

1.1.5 Biosynthesis of triacylglycerol

TAG is a major storage compound in plant seeds and represents up to 60 % of the seed dry weight. Studies conducted by Shockey and co-workers could show that specific ER regions are dedicated to TAG synthesis and that the required enzymes are assembling in these subdomains (Shockey et al., 2006). The traditional view of TAG formation (Figure 1) shares some common features with the biosynthesis of phospholipids via the "eukaryotic" pathway (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). PA is built up at the ER from G3P by sequential acyl-CoA-dependent GPAT and LPAAT activity (Stymne, 1987). Then, PA is dephosphorylated by PA phosphatase (PAP) and thereby converted into DAG. Alternatively, DAG can also be produced from MAG by an acyl-CoA:monoacylglycerol acyltransferase (MGAT), as it could be shown for peanut (*Arachis hypogaea*) cotyledons by Tumaney and co-workers (Tumaney et al., 2001).

The fatty acid composition of DAG can be affected not only by the previously mentioned acyltransferases but also by various remodelling processes. DAG-DAG transacylase (DDT) is an example for direct influence because it transfers acyl chains from one DAG molecule onto another and thereby produces MAG and TAG, as it could be shown for castor bean (*Ricinus communis*) (Lehner and Kuksis, 1996) and safflower (*Carthamus tinctorius)* (Stobart et al., 1997). Also, the CDP-choline:diacylglycerol cholinephosphotransferase (CPT) can convert PC into DAG and thereby provides a mechanism for synthesis of polyunsaturated DAG species. Fatty acids in this way can first be desaturated while bound to PC and are then transferred into the DAG pool (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). In a similar manner, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) transfers the headgroup of PC onto DAG and thus directly changes fatty acid composition in the DAG pool of the cell (Lu et al., 2009a). The remodelling events between DAG and PC currently become more and more important because recent studies could show that most of the newly synthesized fatty acids from the plastid are first transferred onto PC before they reach other glycerolipid species by acyl editing events at both *sn*-1 and *sn*-2 positions (Bates et al., 2007; Bates et al., 2009). These editing events can for example be mediated by the action of acyl-CoA:lyso-phosphatidylcholine acyltransferase (LPCAT). This enzyme transfers acyl chains from the *sn*-2 position of PC onto CoA and *vice versa* (Stymne and Stobart, 1984). Both of these pools are used as raw material for neutral lipid synthesis.

The last step of TAG biosynthesis is carried out by two different enzymatic activities (Figure 1). Acyl-CoA-dependent TAG formation is performed by acyl-CoA:diacylglycerol acyltransferase (DGAT) (Ohlrogge and Browse, 1995). This enzyme transfers an acyl chain from the CoA pool onto DAG and thereby produces TAG and free CoA. Phospholipid:diacylglycerol acyltransferase (PDAT), on the other hand, mediates acyl-CoA-independent TAG synthesis by transferring an acyl chain from the PC

pool onto DAG and in turn releasing TAG and lyso-PC (Dahlqvist et al., 2000). Upon formation at the ER, TAG is then accumulating in phospholipid monolayer-enclosed compartments named oil bodies (Schwarzenbach, 1971; Stobart et al., 1986; Murphy and Vance, 1999). These oil bodies are approximately 1 µm in diameter and contain a number of important structural proteins, for example oleosins, at their surface (Kim et al., 2002). They are the storage rooms for newly synthesized TAGs until they are accessed by lipases upon reserve remobilisation.

The seed oils of some plant species are enriched in unusual fatty acids that have special characteristics like particular functional groups. These fatty acids are normally not found in the membrane lipids of the respective species (Millar et al., 2000). Castor bean oil, for example, is rich in ricinoleic acid (12-hydroxy-18:1(n-9)) which accounts for up to 90 % of total fatty acids in the seed oil. It is produced at the ER while bound to the *sn*-2 position of PC and then transported by highly efficient acyltransferase activities into the TAG fraction (Kroon et al., 2006).

Due to the particular importance of acyltransferases in the process of glycerolipid biosynthesis, the following section is dedicated to the presentation of some important representatives of this group of enzymes.

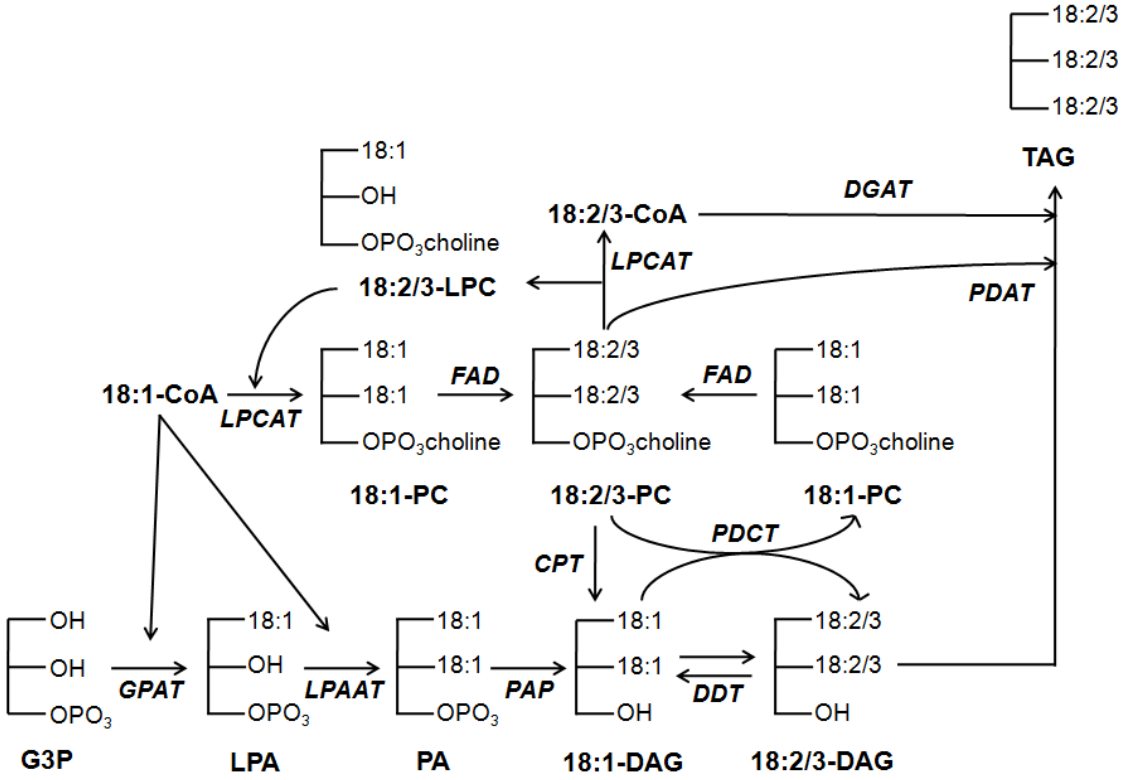

Figure 1. Current TAG biosynthesis model

Abbreviations, in alphabetical order: CoA, Coenzyme A; CPT, CDP-choline:diacylglycerol cholinephosphotransferase; DAG, diacylglycerol; DDT, diacylglycerol:diacylglycerol acyltransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase; FAD, fatty acid desaturase; G3P, Glycerol-3-Phosphate; GPAT, acyl-CoA:glycerol-3-phosphate acyltransferase; LPA, lyso-phosphatidic acid; LPAAT, acyl-CoA:lyso-phosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; LPCAT, acyl-CoA:lyso-phosphatidylcholine acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; TAG, triacylglycerol. Not depicted is the synthesis of DAG from monoacylglycerol (MAG) via acyl-CoA:monoacylglycerol acyltransferase (MGAT). Explanations are given in the text (section 1.1.5). Figure extended on the basis of (Lu et al., 2009a).

1.2 Acyltransferases

1.2.1 Acyl-CoA:lyso-phosphatidylcholine acyltransferases (LPCATs)

LPCATs are important players in the process of acyl editing by mediating the transfer of acyl chains between the *sn*-2 position of PC and CoA pool (Figure 1). Stymne and Stobart identified LPCAT activity in microsomal preparations of rat lung and safflower cotyledons already in the 1980s (Stymne and Stobart, 1984, 1985). However, the first cloning and biochemical characterization of a cDNA encoding a LPCAT from mouse was published only recently (Chen et al., 2006; Nakanishi et al., 2006). These studies showed that LPCAT activity is essential for the synthesis of di-16:0-PC for lung surfactant. In 2007, several studies documented that the yeast gene *YOR175C*, also known as *acyltransferase for lyso-PE* (*ALE1)*, is encoding an acyl-CoA-dependent LPLAT protein capable of acylation of lyso-PC but also of other substrates like lyso-PA, lyso-PE, lyso-PG, lyso-PS and lyso-PI (Benghezal et al., 2007; Chen et al., 2007; Jain et al., 2007; Riekhof et al., 2007; Tamaki et al., 2007). The enzyme was shown to accept substrates with two to 20 carbon atoms and preferred unsaturated acyl chains with 16 to 20 carbon atoms (Tamaki et al., 2007). It was found to be highly enriched in the mitochondria-associated ER membrane (Riekhof et al., 2007) and was classified as member of the membrane-bound O-acyltransferase superfamily (Hofmann, 2000). This superfamily contains several acyltransferases with limited sequence similarity in their putative active site region (Stahl et al., 2008). It exhibits two characteristic amino acids, the first being either histidine or asparagine, the second always being histidine, which is located in the center of a longer hydrophobic stretch. Both amino acids are possibly essential for the catalytic activity (Hofmann, 2000). The first plant LPCATs were identified in 2008 by Ståhl and co-workers in *Arabidopsis*. Biochemical analyses revealed broad specificity in LPLAT activities for both enzymes, encoded by *At1g12640* and *At1g63050* (Stahl et al., 2008).

Recently, Dr. M. Wagner identified nucleotide sequences for putative LPCATs in the microalgae *Ostreococcus tauri* and *Ostreococcus lucimarinus*. The enzyme from the latter-mentioned species was found to partially complement a yeast mutant deficient in endogenous LPCAT activity (Wagner, 2008).

1.2.2 Acyl-CoA:diacylglycerol acyltransferases (DGATs)

DGATs are an essential part of the conventional TAG formation pathway by mediating the transfer of acyl chains from the CoA pool onto the *sn*-3-position of DAG (Figure 1). In *Arabidopsis*, the *tag1-1* (AS11) mutant derived from an ethyl methanesulfonate mutagenesis screen was identified in 1995. Mutant seeds revealed reduced levels of 18:1 and 20:1 and a concomitant accumulation of ALA. Furthermore, overall seed TAG content was reduced and seed development was delayed. The authors assumed a

reduced DGAT activity, which leads to higher substrate levels for desaturation (Katavic et al., 1995). In subsequent studies, the corresponding cDNA sequence encoded by the gene *At2g19450* and named *TAG1*, was isolated, expressed in yeast or insect cells and biochemically analyzed by different groups (Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999). Since then, several DGAT1 enzymes have been identified in various plants like tobacco (*Nicotiana tabacum*), rapeseed (*Brassica napus*), burning bush (*Euonymus alatus*), castor bean, soybean (*Glycine max*), tung tree (*Vernicia fordii*), nasturtium (*Tropaeolum majus*) and *Echium pitardii* (Bouvier-Nave et al., 2000; Nykiforuk et al., 2002; He et al., 2004; Milcamps et al., 2005; Shockey et al., 2006; Wang et al., 2006; Xu et al., 2008; Mañas-Fernández et al., 2009).

In 2001, Lardizabal and co-workers identified two cDNAs encoding putative DGATs from the oleaginous fungus *Mortierella ramanniana*, expressed them in insect cells and assayed cell membranes for DGAT activity. With these experiments they could verify DGAT activity of the encoded proteins. Their sequences, however, were different from the previously described DGATs and thus were classified as DGAT2 enzymes. Homologues of these enzymes were found to be present in fungi, plants and mammals (Lardizabal et al., 2001). In *Saccharomyces cerevisiae*, the gene *YOR245c*, also named *DGA1*, also encodes a DGAT2-like protein. This was found to be the major player for TAG formation in yeast (Sorger and Daum, 2002). Recently, a DGAT2 protein was also identified in castor bean and its encoding gene was shown to be stronger expressed in seeds than the *R. communis* DGAT1 gene. The authors thus concluded an important role of DGAT2 for seed TAG synthesis (Kroon et al., 2006). Shockey and co-workers achieved similar results in tung tree when they compared expression of DGAT1 and DGAT2 genes in this organism. Furthermore, they found out that both enzymes produced different TAG species and were located in distinct ER regions which were not overlapping (Shockey et al., 2006).

Whereas DGAT1 and DGAT2 enzymes are membrane-bound proteins, a third independent cytosolic DGAT enzyme, designated as DGAT3, could be identified in developing peanut cotyledons by Saha and co-workers (Saha et al., 2006). This soluble protein shares more sequence similarities with bacterial bifunctional wax ester synthase (WS)/DGAT enzymes than with DGAT1 and DGAT2 proteins. These can use both fatty alcohols and DAG as acyl acceptor. The presence of bifunctional WS/DGAT proteins has been reported for the bacterium *Acinetobacter* (Kalscheuer and Steinbüchel, 2003; Stoveken et al., 2005) as well as for plants (King et al., 2007; Li et al., 2007).

Very recently, the first microalgal DGATs were identified from *O. tauri* and characterized by Dr. M. Wagner. The sequences were named *OtDGAT2A* and *OtDGAT2B* according to their structural similarity to other DGAT2 sequences. The protein encoded by *OtDGAT2B* revealed much higher activity in yeast and accepted saturated as well as mono- and polyunsaturated acyl-CoAs as substrates (Wagner, 2008).

1.2.3 Phospholipid:diacylglycerol acyltransferases (PDATs)

PDATs mediate TAG formation by transfer of acyl chains from the *sn*-2 position of phospholipids like PE and PC onto the *sn*-3 position of DAG (Figure 1). Acyl-CoA-independent TAG synthesis in plants was observed for the first time in microsomal preparations from different oil seeds (Stobart et al., 1997; Dahlqvist et al., 1998). The responsible enzymatic activity was initially discovered a few years later in *S. cerevisiae* (Dahlqvist et al., 2000; Oelkers et al., 2000). The yeast gene *YNR008w* was demonstrated to encode a PDAT protein structurally similar to mammalian lecithin-cholesterol acyltransferases. Therefore, the gene was designated as *LCAT-related protein 1* (*LRO1)*. The activity of the respective PDAT enzyme depended on the headgroup of the phospholipid acyl donor, the transferred acyl chain and the acyl chains of the acyl acceptor DAG (Dahlqvist et al., 2000). Complete knock-out of the gene led to a strong decrease in TAG content in yeast, whereas over-expression led to an increase of PDAT activity in microsomal assays (Oelkers et al., 2000). Dahlqvist and co-workers also performed *in vitro* studies with microsomal preparations from sunflower (*Helianthus annuus*), castor bean and *Crepis palaestina*. These showed different substrate preferences for the transferred acyl chains which also reflected the TAG composition of the respective plant species (Dahlqvist et al., 2000). In 2004, the first plant PDAT protein, encoded by the gene *At5g13640*, was identified in *Arabidopsis*. Its enzymatic activity was verified by over-expression of the corresponding cDNA *in planta* and subsequent *in vitro* tests with microsomal preparations from different plant tissues. AtPDAT1p was shown to use different phospholipids as acyl donors and had a three-fold preference for the *sn*-2 position over the *sn*-1 position of phospholipids. Acyl groups containing ten to 22 carbon atoms were transferred but, surprisingly, the highest activity was found for acyl chains containing double bonds, epoxy or hydroxyl groups (Stahl et al., 2004). In a complementary approach, Mhaske and co-workers characterized an *Arabidopsis* knock-out line for *At5g13640* and investigated fatty acid content and composition of seeds in comparison to wild type seeds. They could not find any differences and thus concluded that AtPDAT1p does not play an important role for TAG synthesis in seeds (Mhaske et al., 2005). This idea, however, was disproven by a recent study from Zhang and co-workers. They showed that silencing of *AtPDAT1* via RNAi in the *tag1-1* background or *vice versa* silencing *AtDGAT1* in the *atpdat1* background led to a 70-80 % decrease in seed oil, furthermore to pollen sterility and disruption of embryo development. From these data, the authors concluded an overlapping effect of AtDGAT1p and AtPDAT1p in seed oil TAG synthesis (Zhang et al., 2009).

In 2008, a microalgal putative PDAT from *O. tauri* was identified by Dr. M. Wagner. The encoded protein was shown to complement a yeast mutant deficient in endogenous TAG formation upon addition of polyunsaturated fatty acids of the (n-3)- or the (n-6)-series with 18 or 20 carbon atoms (Wagner, 2008).

1.2.4 Biotechnological engineering of plants using acyltransferases

The first reports on the over-expression of acyltransferase sequences in seeds in order to change seed lipid metabolism of plants were published in the mid-90s. In these studies, sequences coding for LPAATs from two meadowfoam species (*Limnanthes alba alba* and *Limnanthes douglasii*) were expressed in rapeseed. This resulted in altered proportions of *sn*-2 erucic acid in seed oil of the host plant and thus influenced stereochemical composition of the TAG fraction (Lassner et al., 1995; Brough et al., 1996). Later expression experiments with a yeast LPAAT sequence *in planta* not only changed the composition of TAGs, but also the total seed oil content. The authors of this study constitutively expressed the sequence for the yeast LPAAT sequence *SLC1* in *Arabidopsis* and rapeseed, which resulted in an 8-48 % increase in seed oil content. Furthermore, proportions and amounts of very long-chain fatty acids in the TAG fraction, especially at the *sn*-2 position of TAG, were strongly enhanced (Zou et al., 1997).

However, most reports in the past have dealt with the implementation of DGAT activities into *Arabidopsis* or oilseed crops. Jako and co-workers in 2001 were the first ones, who over-expressed the sequence encoding AtDGAT1p in *Arabidopsis* seeds. In correlation with the measured expression levels, this positively influenced oil deposition and average seed weight (Jako et al., 2001). In subsequent studies using a similar approach, increases in the oil content could be achieved in soybean, maize (*Zea mays*) and rapeseed by over-expression of either foreign or endogenous DGAT sequences (Lardizabal et al., 2008; Weselake et al., 2008; Zheng et al., 2008; Taylor et al., 2009). Recently, also the production of the uncommon fatty acid ricinoleic acid in the host plant *Arabidopsis* could be enhanced by co-expression of castor bean sequences encoding fatty acid hydroxylase and DGAT activity from 17-30 % compared to the expression of the fatty acid hydroxylase sequence alone (Burgal et al., 2008).

Up to now, there is only one study, which reports on the implementation of a LPLAT activity into the seed metabolism of a host plant. Wu and co-workers established VLCPUFA production in Indian mustard (*Brassica juncea)* and in this context also introduced a putative LPLAT sequence from *Thraustochytrium sp.* into plants. *Thraustochytrium sp.* is rich in docosahexaenoic acid (DHA, 22:6(n-3)) (Lewis et al., 1999). The enzyme was therefore believed to have a preference for VLCPUFAs and might enhance product yields in the host plant. However, it is not clear whether expression of the putative acyltransferase sequence had any effect (Wu et al., 2005).

1.3 Very long-chain polyunsaturated fatty acids (VLCPUFAs)

Important representatives of this group of fatty acids are the (n-6)-fatty acid arachidonic acid (20:4(n-6), ARA) as well as the (n-3)-fatty acids eicosapentaenoic acid (20:5(n-3), EPA) and DHA. All of them are depicted with their respective precursors in Figure 2. The nomenclature of the fatty acids is derived from the position of the first double bond proximal to the methyl end of the molecule. Is this double bond at the sixth position, the respective fatty is ranked among the (n-6)-group. In case the double bond is at the third position, the fatty acid is part of the (n-3)-series. The alternative Δ-nomenclature in Figure 2 indicates the double bond positions counted from the carboxyl end of the molecule.

VLCPUFAs are important membrane constituents and essential for example during fetal development as well as for proper retina and brain function (Carlson et al., 1993; Crawford et al., 1997; Giusto et al., 2000). Furthermore, VLCPUFAs serve important functions as cellular messengers. ARA is the precursor for thromboxanes, leukotrienes and prostaglandins which are activating inflammation processes. EPA and DHA can be converted for example into resolvins, which have anti-inflammatory effects (Hwang, 2000; Funk, 2001; Jump, 2002). The derivatives of (n-3)-VLCPUFAs are known to have health-beneficial effects like protection of the cardiovascular system or reduction of inflammatory processes when taken up in sufficient amounts (Demaison and Moreau, 2002; Simopoulos, 2002; Calder, 2004).

Humans, like all mammals, lack Δ12- and Δ15-desaturases to convert oleic acid into LA and ALA, respectively (Nakamura and Nara, 2003). Therefore, they cannot synthesize these fatty acids de *novo*, but have to take them up with their nutrition as essential precursors for VLCPUFAs (Goyens et al., 2006). In addition, also the direct dietary supply with VLCPUFAs is crucial because conversion rates from LA and ALA into their respective very long-chain derivatives is rather low in men (Gerster, 1998; Pawlosky et al., 2001; Brenna, 2002; Goyens et al., 2006). At present, the ratio between (n-6)- and (n-3)-fatty acids in the Western diet is much too high. Thus, especially the direct uptake of (n-3)-VLCPUFAs would act towards the undersupply with these fatty acids and would lower the (n-6) over (n-3) ratio (Goyens et al., 2006; Simopoulos, 2006).

Marine microalgae are the main primary producers of VLCPUFAs and constitute the base of the aquatic food chain. That is why currently oily sea fish, like tuna, salmon or herring, is the most important dietary VLCPUFA source for humans. However, due to problems like overfishing (Naylor et al., 2000) and pollution of the sea (Hites et al., 2004), research for sustainable production of VLCPUFAs, for example in oilseed crops, is ongoing and was reviewed recently in (Venegas-Calerón et al., 2010).

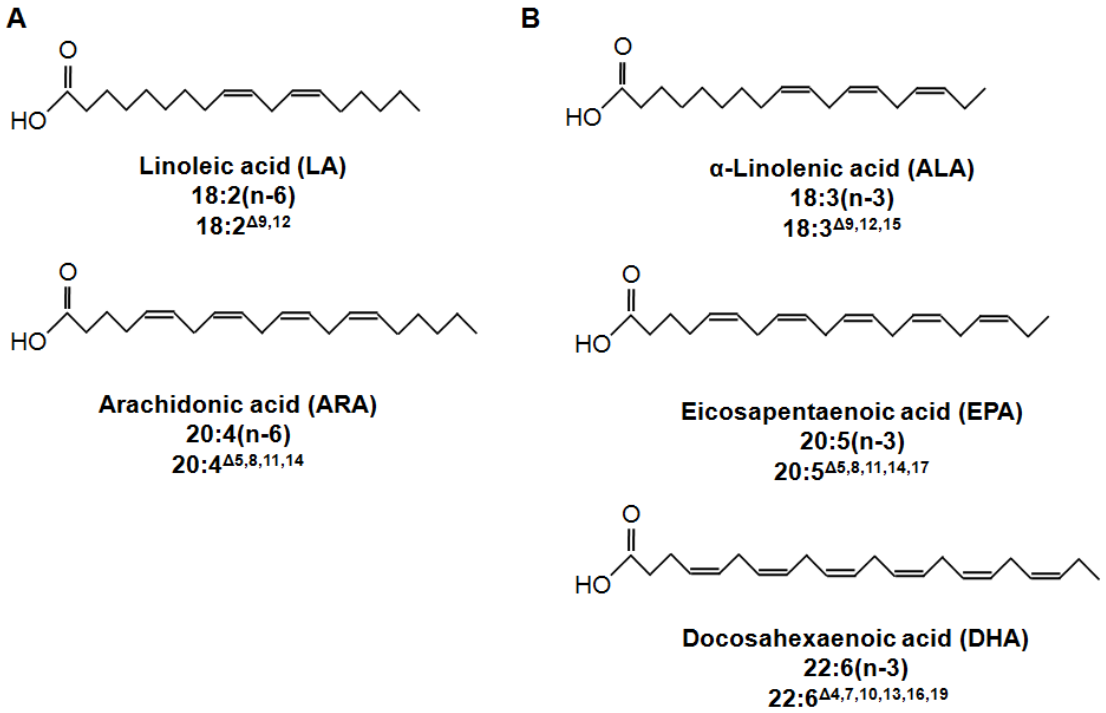

Figure 2. Molecular structures of important VLCPUFAs and their precursors
(A) (n-6)-(VLC)PUFAs (B) (n-3)-(VLC)PUFAs

1.3.1 Biosynthesis of VLCPUFAs

Synthesis of VLCPUFAs is accomplished via two completely different systems. On the one hand, there is the oxygen-independent polyketide synthase (PKS)-like system found in marine bacteria and eukaryotes (Jenke-Kodama et al., 2005). It produces VLCPUFAs *de novo* by combined fatty acyl chain elongation, *trans-cis* isomerisation and enoyl reduction. Acetyl-CoA and malonyl-CoA are used as building blocks for these reactions and the whole system has a modular composition (Metz et al., 2001; Kaulmann and Hertweck, 2002). The synthesis of VLCPUFAs by the PKS-like system releases only trace amounts of intermediates which hints at a highly efficient metabolic flux (Truksa et al., 2009). On the other hand, there is the VLCPUFA biosynthesis system consisting of elongases and desaturases, which is found in marine microalgae as well as in some bacteria, fungi, non-flowering plants like mosses and animals. In Figure 3, the pathways based on these enzymatic activities are illustrated by using the Δ-nomenclature for fatty acids in order to be able to pursue the subsequent steps of desaturation and elongation. ARA is synthesized via the (n-6)-pathway from LA by sequential Δ6-desaturation, Δ6-elongation and Δ5-desaturation. Starting from ALA, but going through the same enzymatic steps, EPA is produced via the (n-3)-pathway. DHA is then built up by Δ5-elongation and Δ4-desaturation of EPA. Both (n-6)- and (n-3)-pathways are interconnected by Δ15- or Δ17-desaturases that convert (n-6)-substrates into (n-3)-substrates (Pereira et al., 2004a; Damude et al., 2006). In addition to the (n-6)- and (n-3)-pathways, also other variations exist. In the Δ8-pathway, LA or ALA are

processed by a Δ9-elongase and are then further metabolized into ARA and EPA by sequential Δ8- and Δ5-desaturation (Wallis and Browse, 1999; Qi et al., 2002; Sayanova and Napier, 2004). According to (Lang, 2007), there may be also a Δ3-pathway, in which 18:4(n-3) is desaturated at the Δ3-position and then elongated to EPA. However, to date no desaturase with Δ3-specificity could be identified. Further, the "Sprecher" pathway found exclusively in mammals is a variation to the conventional pathway of DHA synthesis from EPA. It proceeds from docosapentaenoic acid (22:5(n-3), DPA) via Δ7-elongation and Δ6-desaturation to tetracosahexaenoic acid (24:6(n-3)), which is then processed via regulated peroxisomal β-oxidation into DHA (Voss et al., 1991; Sprecher et al., 1995; Sprecher et al., 1999; Sprecher, 2000). Genes encoding desaturases or elongases have been cloned from various organisms and functionally tested in plants (reviewed by (Truksa et al., 2009; Venegas-Calerón et al., 2010)).

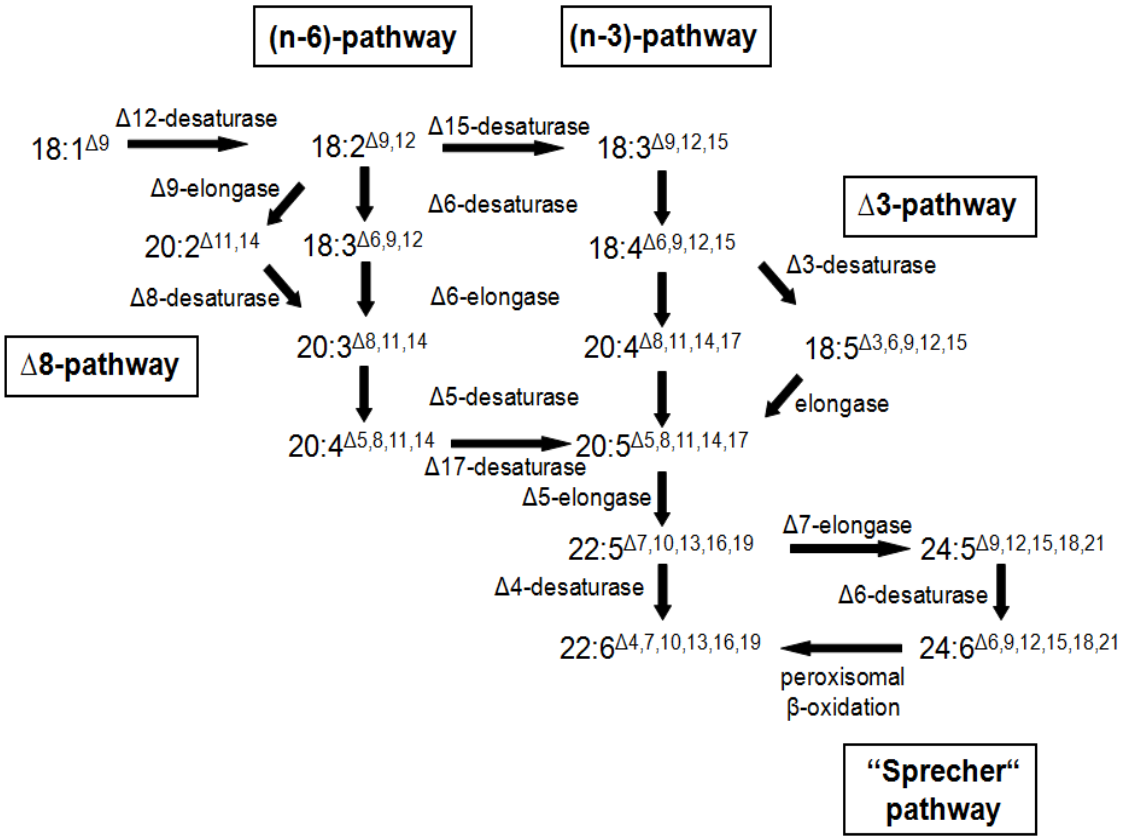

Figure 3. VLCPUFA biosynthesis pathways

(n-6)- and (n-3)-pathway as well as the Δ3-pathway and "Sprecher" pathway are illustrated. Furthermore, the alternative Δ8-pathway, which can also start from $18:3^{\Delta 9,12,15}$ and results in $20:4^{\Delta 8,11,14,17}$, is depicted. Figure taken from (Hoppe, 2008).

1.3.2 Enzymatic activities during VLCPUFA biosynthesis

1.3.2.1 Desaturases

Fatty acid desaturases are non-heme metalloenzymes. Their active site contains a diiron center (Oshino et al., 1966; Nagai and Bloch, 1968; Jaworski, 1974; Strittmatter et al., 1974) and all of them share similarities regarding the stereochemistry of hydrogen abstraction (Shanklin and Cahoon, 1998; Sperling et al., 2003). All desaturases require oxygen (Bloomfield and Bloch, 1960; Mudd and Stumpf, 1961) and a short electron transport chain for their enzymatic activity (Fulco, 1974). In the plastid, ferredoxin is used as electron carrier for the supply with electrons either from nicotinamide adenine dinucleotide phosphate (Nagai and Bloch, 1966; Schmidt and Heinz, 1990; Wada et al., 1993) photosystem I (Jacobson et al., 1974) for the desaturation reaction. In the ER, cytochrome b_5 acts as electron carrier and receives electrons from nicotinamide adenine dinucleotide (Spatz and Strittmatter, 1971; Dailey and Strittmatter, 1979; Hackett and Strittmatter, 1984). The cytochrome b_5 can either exist as separated unit or it is already fused to the desaturase, as it is the case for so-called front-end desaturases (Sperling and Heinz, 2001).

Desaturases can be divided into two classes (Shanklin and Cahoon, 1998; Sperling et al., 2003). One class is constituted by soluble, acyl-ACP-dependent desaturases, which are located in the plastid (Bloomfield and Bloch, 1960) and have been extensively studied in the past (reviewed by (Shanklin and Cahoon, 1998)). The second class of desaturases consists of integral membrane proteins, which are difficult to access by crystallization and therefore have been analyzed by mutagenesis experiments, biochemical topology studies and comparison with related enzymes (Prasad et al., 1980; Stukey et al., 1990; Broun et al., 1998; Diaz et al., 2002; Dyer et al., 2002; Man et al., 2006b). Desaturases of this class, which also includes those desaturases required for VLCPUFA biosynthesis, contain three typical histidine boxes involved in coordination of the diiron center with consensus sequences of $HX_{(3-4)}H$, $HX_{(2-3)}HH$ and $H/QX_{(2-3)}HH$ (Shanklin and Cahoon, 1998). All of the histidines in this tripartite motif are necessary for catalysis (Shanklin et al., 1994). The histidine boxes are, as well as the amino- and the carboxyl-terminus of the desaturase, oriented towards the cytosolic site. From this fact it can be concluded that desaturation takes place in the cytosol (Shanklin and Cahoon, 1998).

Some of the membrane-bound desaturases involved in VLCPUFA biosynthesis are directly fused to their electron donor cytochrome b_5, as mentioned above. Amino-terminal fusions can be found in front-end desaturases from algae and mosses, carboxyl-terminal fusions are common for desaturases from fungi and yeast (Stukey et al., 1990; Sayanova et al., 1997; Itoh et al., 1998; Sperling et al., 2003). The HPGG motif forms the core of the heme-binding domain in cytochrome b_5, is highly conserved and therefore can be used as indicator for the hemeprotein (Lederer, 1994).

Membrane-bound desaturases, neglecting some bifunctional enzymes (Hastings et al., 2001; Damude et al., 2006; Sayanova et al., 2006; Hoffmann et al., 2007), have distinct regio- and substrate-specificities (Shanklin and Cahoon, 1998). They can be classified into different subtypes concerning their location of double bond formation (Heinz, 1993). Front-end desaturases for example introduce new double bonds always between the carboxyl end of the fatty acid and an already existing double bond (Aitzetmüller and Tsevegsüren, 1994). They can have Δ4-, Δ5-, Δ6- or Δ8-specificity (Domergue et al., 2005) and contain an H to Q substitution in their third histidine box, which is crucial for enzyme activity (Sayanova et al., 1997; Michaelson et al., 1998; Napier et al., 1998; Sayanova et al., 2001).

In contrast to the before-mentioned type, there are also the so-called ω-desaturases, which position the double bond completely new or between an already existing double bond and the methyl end of the molecule. They have Δ12- or Δ15-specificity and share a conserved HECGH motif (first histidine box) and two HX_2H_2 motifs (second and third histidine box) (Pereira et al., 2004a).

Most membrane-bound desaturases from the plant kingdom are acting on substrates that are bound to complex lipids like for example PC (Stymne and Stobart, 1986; Jackson et al., 1998; Galle-Le Bastard et al., 2000; Domergue et al., 2003) or MGDG (Norman et al., 1991). In contrast, mammalian desaturases are CoA-dependent (Okayasu et al., 1981; Irazú et al., 1993; Domergue et al., 2003), as are some recently disco-vered microalgal desaturases from *O. tauri* and *Mantoniella squamata* (Domergue et al., 2005; Hoffmann et al., 2008).

1.3.2.2 Elongases

Elongases are membrane-bound enzyme complexes at the ER, which condense C16 or C18 fatty acids with malonyl-CoA and thereby elongate them by two-carbon chain units. They are composed of four different proteins, which perform the sequential reactions of condensation of the acyl chain with malonyl-CoA (β-ketoacyl-CoA synthase, KCS), reduction (β-ketoacyl-CoA reductase, KCR), dehydration (hydroxyacyl-CoA dehydratase, HCD) and again reduction (enoyl-CoA reductases, ECR) (Fehling et al., 1992). The condensing enzyme is believed to be the determinant of substrate specificity and reaction velocity (Venegas-Calerón et al., 2010) and in previous studies, expression of KCS sequences was shown to be able to reconstitute heterologous elongation activity by interaction of the foreign KCS with the endogenous KCR, HCD and ECR activities (Millar and Kunst, 1997; Beaudoin et al., 2000; Parker-Barnes et al., 2000). The introduction of a foreign elongase into a host organism thus always refers to the respective KCS protein (Venegas-Calerón et al., 2010). In contrast to most plant desaturases, elongases act in an acyl-CoA-dependent way (Domergue et al., 2003; Jakobsson et al., 2006).

1.3.3 VLCPUFA production in plants

Currently, oily sea fish and marine microalgae are the main direct nutritional sources of VLCPUFAs for humans. Both of these sources, however, comprise major disadvantages. Fish stocks are declining all over the world and the problem of overfishing will even get worse in the future due to a growing world population (Naylor et al., 2000). Furthermore, residual fish stocks are contaminated with pollutants like methyl mercury and organochlorines, which make them useless for human consumption (Hites et al., 2004). Fish farming has been considered as an adequate solution to this problem in the past, but has led to severe environmental destruction world-wide (Pauly et al., 2002). Also, aquaculture requires more input of VLCPUFAs than it gives rise to (Venegas-Calerón et al., 2010). Cultivation of VLCPUFA-producing microalgae, on the other hand, requires large fermenters and is difficult to scale up (Lee, 2001). It is sensitive to changes in the power supply and holds a big environmental footprint (Venegas-Calerón et al., 2010). Taken together with an elaborate oil extraction procedure, the whole process is a very expensive and laborious approach to obtain the valuable products. For these reasons, much research has been conducted on the synthesis of VLCPUFAs in oleaginous organisms like annual oilseed crops during the last two decades. Evident advantages of VLCPUFA production in plants in this connection are the already existing infrastructures for plant cultivation and seed oil extraction with lower costs and efforts in comparison to algae cultivation and microbial oil extraction (Alonso and Maroto, 2000; Galili et al., 2002; Thelen and Ohlrogge, 2002).

Because most oilseed plants naturally synthesize only the VLCPUFA precursor fatty acids LA and ALA, several sequences encoding enzymes for the whole VLCPUFA biosynthetic pathway have to be transferred from suitable gene donors into plants and need to be expressed in a coordinated way in the desired plant tissue. Several studies were directed towards this aim in the previous years. The main findings will be presented in the following section.

Concerning the above-mentioned PKS-like system for VLCPUFA production in marine bacteria, some cloning approaches for enzymes acting in this system exist and recently, in *Arabidopsis* also small amounts of DHA could be produced (Metz et al., 2006). One major disadvantage of the utilization in plants might be that the PKS-like system produces free fatty acids that would require activation for lipid incorporation in seeds (Metz et al., 2009).

Regarding the alternative system employing concerted desaturation and elongation reactions for heterologous VLCPUFA production in plants, first results were published amongst others by Abbadi and co-workers (Abbadi et al., 2004). Seed-specific expression of sequences encoding a Δ6-desaturase and a Δ5-desaturase from the diatom *Phaeodactylum tricornutum* (*Ptd6* and *Ptd5*, respectively) (Domergue et al., 2002) together with the sequence for the Δ6-elongase from the moss *Physcomitrella patens* (*PSE1*) (Zank et al., 2002) was performed in tobacco high in LA and linseed (*Linum usitatissimum*) high in ALA. With less than 1 %, only very low amounts of ARA and EPA were detectable. However, an accumulation of the Δ6-desaturation products

about 25 % was observed in the total lipids. At the same time, very low levels of this first intermediate of the pathway were found in the acyl-CoA pool of transgenic seeds. Taking together these data and the results from previous studies, the authors concluded that the lack of Δ6-desaturation products in the CoA pool might be due to either direct incorporation of intermediates into the TAG fraction or an insufficient substrate transfer between PC and CoA pool (Domergue et al., 2003; Abbadi et al., 2004).

An alternative approach for VLCPUFA production in plants was chosen by (Qi et al., 2004). In this study, the Δ8-pathway was established in *Arabidopsis* by constitutive expression of a Δ9-elongase sequence from *Isochrysis galbana* (Qi et al., 2002), a Δ8-desaturase sequence from *Euglena gracilis* (Wallis and Browse, 1999) and a Δ5-desaturase sequence from *Mortierella alpina* (Knutzon et al., 1998). This approach circumvented one shuttling step between PC and CoA pool and with 7 % ARA and 3 % EPA in leaves led to higher product yields compared to those achieved by Abbadi and co-workers (Qi et al., 2004).

Substantial increases in product levels as well as DHA production in plants could be attained by Kinney et al. in the same year (Kinney et al., 2004). Soybean somatic embryos were transformed with one vector containing sequences from *M. alpina* encoding the enzymatic activities for EPA production and in addition with a second vector containing sequences for a Δ17-desaturase from *Saprolegnia diclina* (Pereira et al., 2004a), a Δ5-elongase sequence from *Pavlova salina* (Pereira et al., 2004b) and a Δ4-desaturase sequence from *Schizochytrium aggregatum*. Up to 3.3 % DHA was found in the total fatty acids and up to 20 % EPA was detectable in seeds from plants deriving from transformation with the EPA construct (Kinney et al., 2004).

In a study conducted by (Wu et al., 2005) in Indian mustard, a series of binary vectors containing three to nine genes all controlled by the same seed-specific promoter was used for the stepwise addition of enzymatic activities to be able to follow the metabolic flux in plants. ARA yields averaged 18 %, EPA yields were up to 8 % and DHA yields were about 0.2 % of total fatty acids. Wu and co-workers made observations that were similar to the findings described by Abbadi et al. (2004), but could enhance elongation rates by expression of a second elongase sequence. From these data the authors concluded that substrate availability is not the only limiting factor for elongation efficiency (Wu et al., 2005).

Both last-mentioned studies thus led to higher product yields in soybean and Indian mustard compared to linseed and tobacco. Therefore, it can be concluded that the utilized host plant and its endogenous acyltransferase activities have a great impact on successful VLCPUFA production (Napier, 2007a). This fact was also reinforced by complementary experiments conducted by Abbadi and co-workers. In these studies, the same enzymatic activities used before were introduced into a linseed type rich in LA as well as in rapeseed. ARA or ARA and EPA, respectively, accumulated strongly in transgenic seeds. The accumulation of Δ6-desaturation products was lower and the elongation rates were higher compared to the data obtained in tobacco and linseed high in ALA (Abbadi et al., 2007).

In a first approach to enhance VLCPUFA yields in plants by utilization of an exclusively acyl-CoA-dependent pathway, *A. thaliana* was transformed with a construct containing a sequence encoding a bifunctional acyl-CoA-dependent Δ6/Δ5-desaturase from zebrafish (*Danio rerio*) (Hastings et al., 2001) and a Δ6-elongase sequence from *Caenorhabditis elegans* (Beaudoin et al., 2000). Expression led up to 1.6 % ARA and 3.2 % EPA of total fatty acids. Plants were retransformed with a Δ5-elongase and a Δ4-desaturase from *P. salina*, which led up to 0.5 % DHA (Robert et al., 2005). However, the heterologous expression of genes from animals in plants always evokes ethical questions and is not accepted by the general public (Napier, 2007b). Therefore, the utilization of enzymes from the plant kingdom would be favourable. This aim could recently be achieved by (Hoffmann et al., 2008). In this study, sequences encoding acyl-CoA-dependent desaturases could be identified in the microalgal species *M. squamata*. They were co-expressed together with *PSE1* (Zank et al., 2002) in *Arabidopsis* under the control of a seed-specific promoter. These experiments could demonstrate the circumvention of the transfer bottleneck after the first desaturation step, because the accumulation of Δ6-desaturation products could not be observed any more. Due to the low activity of the identified Δ5-desaturase, however, EPA yields were comparable to the ones obtained with the lipid-dependent desaturases, which had also been used by (Abbadi et al., 2004).

In summary, EPA yields achieved by heterologous expression of genes from diverse gene donor organisms are already quite satisfying. For DHA production, however, still some improvements concerning metabolic flux need to be done (Truksa et al., 2009). This could for example be achieved by the identification of an acyl-CoA-dependent Δ4-desaturase, that supersedes the requirement of shuttling between PC and CoA pool also in this last part of the (n-3)-pathway.

1.4 Aims of the project

The experiments in the presented thesis were performed to continue preceding studies on the identification and characterization of enzymes directly or indirectly involved in microalgal VLCPUFA biosynthesis.

In studies conducted by Dr. M. Wagner, different sequences putatively encoding acyltransferases were identified in the microalgal species *O. tauri* and *O. lucimarinus* and characterized in parts (Wagner, 2008). Their substrate specificities as well as their possible interaction should be further investigated by *in vitro* and *in vivo* experiments in yeast.

As described in the previous section, Dr. M. Heilmann could identify two exclusively acyl-CoA-dependent desaturases with Δ6- and Δ5-specificity from *M. squamata* and expressed their sequences together with the elongase sequence *PSE1* in *Arabidopsis.* By this approach, she could establish EPA biosynthesis *in planta* and circumvented the bottleneck between desaturation and elongation described by Abbadi and co-workers (Hoffmann et al., 2008). The next logical step would be the synthesis of DHA by introduction of an additional elongation and a desaturation activity. A few elongases with Δ5-specificity have already been isolated from microalgae (Meyer et al., 2003; Pereira et al., 2004b). Thus, the aims of this work were to identify an acyl-CoA-dependent desaturase with Δ4-specificity, to characterize the enzyme by heterologous expression of its encoding sequence in yeast and to check its suitability for utilization *in planta*.

Last but not least, putative microalgal acyltransferases should be introduced into seed lipid metabolism of wild type and mutant plants to examine their effects *in planta*. Also, VLCPUFA production in plants should be further investigated by co-expression of different desaturase and elongase sequences at hand. VLCPUFA yields should be compared in consecutive transgenic plant generations possessing various combinations of desaturation and elongation activities and the interplay of desaturases and elongases should be compared in different plant species. Furthermore, desaturase, elongase and acyltransferase sequences should be co-expressed in *Arabidopsis* to test possible acyltransferase effects on VLCPUFA yields.

2 Material and Methods

Basic molecular biological and biochemical techniques were performed as described in (Ausubel et al., 1993). For all methods sterile pipet tips and reaction tubes were used. All solutions were set up with double distilled water (ddH_2O) and sterilized by autoclaving them for 15-20 minutes (min) at 120 °C.

2.1 Technical Equipment

ABI PRISM 3100 genetic analyzer	Applied Biosystems (Foster, USA)
Biofuge pico	Heraeus Sepatech (Osterode, Germany)
Centrifuge 5810 R	Eppendorf AG (Hamburg, Germany)
Centrifuge 5415 D	Eppendorf AG (Hamburg, Germany)
Centrifuge 5417 R	Eppendorf AG (Hamburg, Germany)
Chromatogram immersion device III	Camag (Muttenz, Switzerland)
Clean bench Prettl-Telstar Bio-II-A	Telstar (Terrass, Spain)
6890 series gas chromatograph	Agilent (Waldbronn, Germany)
6890 gas chromatograph/ 5973 mass selective detector system	Agilent (Waldbronn, Germany)
Gel detection system IDA	Raytest (Straubenhardt, Germany)
1100 series HPLC system	Agilent (Waldbronn, Germany)
Mastercycler gradient	Eppendorf AG (Hamburg, Germany)
Mastercycler personal	Eppendorf AG (Hamburg, Germany)
Mini-PROTEAN 3 system	Biorad (Hercules, CA, USA)
Mini Trans-Blot cell equipment	Biorad (Hercules, CA, USA)
Optima L-80K ultracentrifuge	Beckman Coulter (Krefeld, Germany)
Optimax Typ TR developer	MS Labware (Wiesloch bei Heidelberg, Germany)
Percival climate chamber	CLF Plant Climatics (Emersacker, Germany)
Phenyl-hexyl column (5 µm, 150 mm x 2 mm)	Phenomenex (Aschaffenburg, Germany)
Phospho- and fluorescence-imager Fuji FLA-3000	Raytest (Straubenhardt, Germany)
TLC heating plate	Camag (Muttenz, Switzerland)
TLC spray cabinet/sprayer	Camag (Muttenz, Switzerland)
Ultrospec 1100 pro	GE Healthcare (Freiburg, Germany)

2.2 Chemicals and Standards

All chemicals, if not mentioned otherwise, were purchased from Sigma (Munich, Germany), Merck (Darmstadt, Germany), Carl Roth & Co. (Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany) or Duchefa Biochemistry (Haarlem, Netherlands). All solvents of high performance liquid chromatography grade for the analytical methods were purchased from Acros (Geel, Belgium) or Baker (Griesheim, Germany). Fatty acid substrates were ordered from Cayman Chemical (Ann Arbor, MI, USA). Fatty acid standards were from Sigma (Munich, Germany) and acyl-CoA standards from Larodan (Malmö, Sweden). Radiolabeled acyl-CoAs were ordered from American Radiolabeled Chemicals (St. Louis, MO, USA).

2.3 Enzymes

Restriction enzymes

Restriction enzymes *AscI, ApaI*, *BamHI, EcoRI*, *HindIII*, *KpnI*, *NheI*, *NotI, SpeI* and *XhoI* were ordered from Fermentas (St. Leon Rot, Germany), restriction enzyme *SbfI* from was from New England Biolabs (Ipswich, UK). All of them were used according to the manufacturer's instructions.

Other enzymes

Advantage PCR Enzyme System	Clontech (Mountain View, CA, USA)
Calf intestine alkaline phosphatase	Fermentas (St. Leon Rot, Germany)
DNAse I	Fermentas (St. Leon Rot, Germany)
TaKaRa ExTaq DNA polymerase	Cambrex BioScience (Potsdam, Germany)
Phusion DNA polymerase	Finnzymes (Espoo, Finland)
T4 DNA ligase	Fermentas (St. Leon Rot, Germany)

2.4 Kits and Systems

ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1	Applied Biosystems, Foster, USA
ECL Western blotting detection reagents and analysis system	GE Healthcare (Freiburg, Germany)
Gateway LR Clonase II Enzyme Mix	Invitrogen (Karlsruhe, Germany)
NucleoSpin Extract Kit	Macherey-Nagel (Düren, Germany)
NucleoSpin Plasmid Kit	Macherey-Nagel (Düren, Germany)
RevertAid H Minus First Strand cDNA synthesis kit	Fermentas (St. Leon Rot, Germany)

2.5 Vectors

Subcloning vectors

pGEM-T	Amp^R (Promega, Madison, WI, USA)
pUC18-Entry2	Amp^R (provided by Dr. E. Hornung)

pUC18-Entry2 contains a multiple cloning site with inserted *ccdB* gene for selection of positive transformants and flanking *attL1* and *attL2* sites for gateway cloning.

Yeast expression vectors

pESC-TRP	Amp^R (Stratagene, La Jolla, CA, USA)
pESC-LEU	Amp^R (Stratagene, La Jolla, CA, USA)
pYES2/CT	Amp^R (Invitrogen, Karlsruhe, Germany)
pUG36	Amp^R (by courtesy of Dr. R. Krick)

pUG36 was created by Dr. U. Güldener and Prof. Dr. J. H. Hegemann (http://mips.helmholtz-muenchen.de/proj/yeast/info/tools/hegemann/gfp.html).

Plant expression vectors

pCAMBIA3300	Kan^R (bacteria), $glufosinate^R$ (plants) (provided by Dr. Andreas Hiltbrunner, ETH Zürich, Switzerland; with modifications according to (Abbadi et al., 2004))
pCAMBIA33.2cGs	Kan^R (bacteria), $glufosinate^R$ (plants) (provided by Dr. E. Hornung)

The pCAMBIA vectors contain the *bar* gene under the control of the constitutive *Cauliflower mosaic virus 35S* promoter. pCAMBIA3300 contains *SbfI* and *AscI* restriction sites. pCAMBIA33.2cGs contains the seed-specific *LeB4* promoter (Bäumlein et al., 1991b) behind *attR1* and *attR2* sites for gateway cloning.

2.6 DNA constructs

Subcloning constructs

Ptd6-PSE1-Ptd5-pUC19-USP-OCS123 (*SbfI*)	provided by Dr. M. Wagner
Msd6-pUC19-USP-OCS123 (*AscI*)	
Msd6-OlLPCAT-pUC19-USP-OCS123 (*AscI*)	
OlLPCAT-pUC19-USP-OCS123 (*AscI*)	

pUC19-USP-OCS123 (Abbadi et al., 2004) with modifications) was used for subcloning of cDNAs that should subsequently be brought into pCAMBIA3300. It contains three different polylinkers between three seed-specific *USP* promoters (Bäumlein et al., 1991a) and three *OCS* terminators (Macdonald et al., 1991). This three-way expression cassette can be cut out of the pUC vector by restriction digest either with *AscI* or *SbfI.*

Yeast expression constructs

OtDGAT2A-pYES2/CT	provided by Dr. M. Wagner (Wagner, 2008)
OtDGAT2B-pYES2/CT	
OtDGAT2C-pYES2/CT	
OlLPCAT-pYES2/CT	
OlLPCAT-V5-pYES2/CT	
DGA1-pYES2/CT	
OtPDAT-pYES2/CT	
AtDGAT1-pBT3N	provided by Dr. M. Heilmann
AtDGAT2-pBT3N	
OtLPCAT-pESC-TRP	
mCherryFAR1-pESC-URA	
WS-mCherryFAR1-pESC-URA	
ALE1-pYES2.1/TOPO	by courtesy of Dr. U. Ståhl (Stahl et al., 2008)
LacZ-V5-pYES2/CT	Invitrogen (Karlsruhe, Germany)

cDNA sequences for *OtDGAT2A*, *OtDGAT2C* and *OlLPCAT* were completely codon-optimized for optimal expression in yeast and *Arabidopsis*. In cDNA sequences for *OtDGAT2B* and *OtPDAT* codons 1-25 were optimized. The process of codon-optimization is described in (Wagner, 2008). In case of *OtLPCAT*, codons 1-14 were optimized.

FAR1 is a fatty acid reductase sequence from mouse (Cheng and Russell, 2004a), WS is a wax synthase sequence from mouse (Cheng and Russell, 2004b).

Plant expression constructs

Msd6-PSE1-Msd5-OtELO5-Egd4-pCAMBIA3300	provided by S. Schlenczek
Ptd6-PSE1-Ptd6-pCAMBIA3300	provided by Dr. M. Heilmann (Hoffmann et al., 2008)

2.7 Antibodies

Primary antibodies

Anti-V5-mouse	Invitrogen (Karlsruhe, Germany)
Anti-GFP-mouse	Covance (Munich, Germany)

Secondary antibodies

Anti-mouse-peroxidase	Sigma (Munich, Germany)

2.8 Software for Sequence Analyses

For identification of new putative desaturase sequences the databases of the National Center of Biotechnology Information (NCBI) were scanned using the Basic Local Alignment Tool (BLAST) algorithm (Altschul et al., 1997) with already known front-end desaturases as queries (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The identified nucleotide sequence was then further analyzed by similarity searches in the NCBI databases using the BLAST program. Hits with experimentally proven substrate specificity were chosen for global pairwise alignment using EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/emboss/align/; needle-algorithm; Blosum62 matrix) (Rice et al., 2000). Multiple alignments of DNA or nucleotide sequences were done using the program MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) (Corpet, 1988). Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html) was then used to display the results. For protein localization predictions, the programs WoLF PSORT (http://www.wolfpsort.org) (Horton et al., 2007) was used, for prediction of chloroplast transit peptides (cTPs), ChloroP1.1 (http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson et al., 1999) was applied. The program ProtScale (http://www.expasy.ch/tools/protscale.html) (Gasteiger et al., 2005) was used to predict the molecular weight of proteins. Phylogenetic trees were generated using the CLUSTALX multiple alignment program (Thompson et al., 1997) and the program Phylip (Felsenstein, 2005). The program TreeIllustrator was used to display the resulting phylograms (Trooskens et al., 2005).

2.9 Organisms

2.9.1 Algae

Ostreococcus lucimarinus (CCMP2972) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, Maine, USA). *Thalassiosira pseudonana* (1020-1b) was provided by the culture collection of algae Göttingen (SAG, Göttingen, Germany).

2.9.2 Bacteria

Escherichia coli XL1blue	Genotype: *endA1, hsdR17, supE44, thi-1, rec A1, gyrA96, relA1, lac, [F´, proAB, lacI^{q}ZΔ15, Tn10(tet^R)]*; Stratagene (La Jolla, CA, USA) (Bullock et al., 1987)
Agrobacterium tumefaciens EHA105	Genotype: pTiBo542ΔT-DNA Rif^R (Hood et al., 1993)
A. tumefaciens C58C1	Genotype: pTiBo542ΔT-DNA Rif^R, derivative of the strain EHA101 (Hood et al., 1993; Hellens et al., 2000), transformed with Ptd6-PSE1-Ptd5-pCAMBIA2200 (Camp^R (bacteria), Kan^R (plants); (Abbadi et al., 2004))

2.9.3 Yeast

S. cerevisiae INVSc1	Genotype: *his3Δ1/his3Δ1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52* (Invitrogen, Karlsruhe, Germany)
S. cerevisiae BY4741	Genotype: *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (Brachmann et al., 1998)
S. cerevisiae BY4741 *ale1Δ*	Genotype: as above; *ale1-Δ::kanMX4* (Euroscarf, Frankfurt, Gemany)
S. cerevisiae BY4741 *lro1Δ dga1Δ*	Genotype: as above; *lro1-Δ::kanMX4 dga1-Δ::natMX4* (provided by Dr. M. Wagner)
S. cerevisiae BY4741 *lro1Δ dga1Δ ale1Δ*	Genotype: as above; *lro1-Δ::kanMX4 dga1-Δ::natMX4 ale1-Δ::hphMX4* (this work)
S. cerevisiae BY4741 *lro1Δ dga1Δ tes1Δ*	Genotype: as above; *lro1-Δ::kanMX4 dga1-Δ::natMX4 tes1-Δ::hphMX4* (this work)

S. cerevisiae W303 H1246	Genotype: *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 are1-Δ::HIS3 are2-Δ::LEU2 dga1-Δ::KanMX4 lro1-Δ::TRP1 ADE2* (Sandager et al., 2002), by courtesy of Prof. Dr. S. Stymne)

2.9.4 Plants

Arabidopsis thaliana L var. Columbia (Col-0)

A. thaliana L var. Columbia (Col-0) *tag1-1* mutant (AS11; (Katavic et al., 1995))

A. thaliana L var. Columbia (Col-0) transformed with
Ptd6-PSE1-Ptd5-pCAMBIA3300
Msd6-PSE1-Msd5-pCAMBIA3300
Otd6-PSE1-Otd5-pCAMBIA3300
provided by Dr. M. Heilmann (Hoffmann et al., 2008)

Camelina sativa L.Crantz subsp. Ligena

2.10 Cultivation conditions

2.10.1 Algae

O. lucimarinus cultures were grown as batch cultures in 200 ml sterile liquid L1 medium, *T. pseudonana* cultures were grown as batch cultures in 200 ml sterile liquid 1/2 SWES medium with 1 % silicate. All cultures were cultivated at 20 °C, 110 µmol photons*m^{-2}*s^{-1} and a day length of 14 hours in a Percival climate chamber (CLF Plant Climatics).

L1 medium (Guillard, 1993)

75 mg/l	$NaNO_3$
5 mg/l	NaH_2PO_4*H_2O
30 mg/l	Na_2SiO_3*9 H_2O
1 ml/l	L1 trace element solution
0.5 ml/l	f/2 vitamin solution

The medium was set up in filtered seawater (Biologische Anstalt Helgoland, Germany) and autoclaved at 120 °C for 20 min. The vitamin solution was added afterwards, the medium was stored at 4 °C.

L1 trace element solution

	Stock solution (g/l)	Quantity
$Na_2EDTA*2\ H_2O$		4.36 g
$FeCl_3*6\ H_2O$		3.15 g
$MnCl_2*4\ H_2O$	178.1	1 ml
$ZnSO_4*7\ H_2O$	23	1 ml
$CoCl_2*6\ H_2O$	11.9	1 ml
$CuSO_4*5\ H_2O$	2.5	1 ml
$Na_2MoO_4*2\ H_2O$	19.9	1 ml
H_2SeO_3	1.29	1 ml
$NiSO_4*\ 6H_2O$	2.63	1 ml
Na_3VO_4	1.84	1 ml
K_2CrO_4	1.94	1 ml

The solution was set up in ddH_2O, autoclaved at 120 °C for 20 min and stored at 4 °C.

f/2 vitamin solution (Guillard and Ryther, 1962)

1 mg/l Cyanocobalamine (vitamin B_{12})
1 mg/l Biotin (vitamin H)
100 mg/l Thiamine chloride (vitamin B_1)
The solution was set up in ddH_2O, filter sterilized and stored at 4 °C.

1/2 SWES medium with 1 % silicate

0.2 g/l KNO_3
20 mg/l $MgSO_4*7\ H_2O$
20 mg/l KH_2PO_4
30 ml/l Soil extract[#]
5 ml/l Micronutrient solution
450 ml/l ddH_2O
455 ml/l Filtered seawater (Biological Institute Helgoland, Germany)
50 ml/l Saturated solution of $Na_2SiO_3*9\ H_2O$

[#]Soil extract was produced from garden soil. The soil was mixed with water in the ratio 1:2 and autoclaved three times at 120 °C for 20 min. The supernatant was filtered through a round filter (Whatman GmbH, Dassel, Germany) and the solution was stored at 4 °C.

The medium was autoclaved at 120 °C for 20 min and cooled down. Then sterile filtrated vitamin B_{12} (5 µg/l) was added. The solution was stored at 4 °C.

Micronutrient solution

I	**Stock solution (g/100 ml)**	**Used volume (ml)**
$ZnSO_4$*7 H_2O	0.1	1
$MnSO_4$*4 H_2O	0.1	2
H_3BO_3	0.2	5
$Co(NO_3)_2$*6 H_2O	0.02	5
Na_2MoO_4*2 H_2O	0.02	5
$CuSO_4$*5 H_2O	0.0005	1
ddH_2O		881
II		
$FeSO_4$*7 H_2O	0.7	
EDTA	0.8	
ddH_2O		100

Both solutions were set up separately in ddH_2O to avoid precipitations. They were autoclaved at 120 °C for 20 min, then combined and stored at 4 °C.

2.10.2 Bacteria

E. coli XL1blue cells were grown at 37 °C in liquid Luria and Bertani (LB) medium while shaking at 200 rpm or on solid LB medium. *A. tumefaciens* cells were grown at 28 °C in liquid YEB medium while shaking at 200 rpm or on solid YEB medium. For the production of solid medium 1.5 % agar (w/v) (Duchefa Biochemistry, Haarlem, Netherlands) was added to liquid medium. All media were set up in ddH_2O and autoclaved at 120 °C for 20 min. For selection purposes different media additives were added.

LB medium (Silhavy et al., 1984)

10 g/l	Peptone
5 g/l	Yeast Extract
10 g/l	NaCl

YEB medium

5 g/l	Beef extract
1 g/l	Peptone
1 g/l	Yeast Extract
5 g/l	Sucrose
5 mM	$MgSO_4$*7 H_2O

Media additives

	Solvent	Stock solution	End concentration
Carbenicillin	ddH_2O	100 mg/ml	100 mg/l
Kanamycin	ddH_2O	50 mg/ml	25 mg/l
Rifampicin	Dimethylsulfoxide (DMSO)	50 mg/ml	50 mg/l
IPTG	ddH_2O	0.1 M	0.2 mM
X-Gal	Dimethylformamide (DMF)	2 % (w/v)	0.004 % (w/v)
Tetracycline	Ethanol	10 mg/ml	10 mg/l

2.10.3 Yeast

All *S. cerevisiae* strains were grown in liquid or on solid YPD or SD medium at temperatures between 16 °C and 30 °C as indicated for each experiment. Liquid cultures were shaken at 150-200 rpm. For production of solid medium 2 % (w/v) agar (Duchefa Biochemistry, Haarlem, Netherlands) were added to liquid medium. All media were set up in ddH_2O and autoclaved at 120 °C for 15 min.

YPD medium (complete medium)

10 g/l	Yeast Extract
20 g/l	Peptone
20 g/l	Glucose Monohydrate

For selection purposes, hygromycin B was added to a final concentration of 300 µg/ml after autoclaving the medium.

SD medium (selection medium)

1.7 g/l	Yeast Nitrogen Base (MP Biomedicals, Heidelberg, Germany)
2.5 g/l	$(NH_4)_2SO_4$

After autoclaving and before use, 2 % (w/v) sugar (sterile filtrated glucose, raffinose or galactose) as well as synthetic complete drop-out medium mix to a final concentration of 1 x were added.

50 x Synthetic complete drop-out medium mix

2 g	Adenine hemisulfate
2 g	L-Arginine HCl
2 g	L-Histidine HCl
2 g	L-Isoleucine
4 g	L-Leucine
2 g	L-Lysine HCl
2 g	L-Methionine
3 g	L-Phenylalanine
2 g	L-Serine
2 g	L-Threonine
3 g	L-Tryptophan
2 g	L-Tyrosine
1.2 g	Uracil
9 g	L-Valine

Substances were mixed, leaving out the respective component for selection, and ground. They were resuspended to a concentration of 100 g/l in sterile ddH_2O and stored at 4 °C.

2.10.4 Plants

2.10.4.1 Surface sterilization of *A. thaliana* seeds

Prior to sterile cultivation of *A. thaliana* plants on plates, seeds were sterilized. For a 150 mm plate, 40 mg of seeds were mixed with 1 ml 6 % (w/v) NaOCl containing 0.1 % (v/v) Triton X-100 in sterile tubes and were incubated on a rocking table for 15 min. Supernatants were discarded and seeds were washed four times with 1 ml of sterile ddH_2O. Afterwards, seeds were resuspended in 6-8 ml 0.1 % (w/v) agar.

2.10.4.2 Cultivation on plates

Surface-sterilized seeds were sown onto plates containing 1/2 Murashige Skoog (MS) medium. For selection of transgenic plants, kanamycin was added to a final concentration of 40 µg/ml after autoclaving. For freshly transformed seeds, also cefotaxime was added to a final concentration of 100 µg/ml to prevent agrobacteria from growing on the plates. Plates were wrapped with 3M micropore tape and then incubated at 23 °C and under constant illumination of 120 µE.

1/2 MS medium

2.2 g/l Murashige Skoog powder

1 % (w/v) Sucrose

7 g/l Microagar

The medium was set up in ddH_2O, adjusted to pH 5.9 with KOH and then autoclaved for 20 min at 120 °C.

2.10.4.3 Cultivation on soil

Prior to usage, soil was incubated for 8 hours at 180 °C to reduce fungal contaminations. *A. thaliana* seeds were sown non-sterile on soil (Frühstorfer Erde Typ EP Nr. 340, Industrie Erdwerk Archut, Lauterbach-Wallenrod, Germany) and incubated for stratification 2-4 days at 4 °C. Afterwards, plants were either cultivated in the greenhouse at a day length of 16 hours and temperatures between 16-22 °C or in the climate chamber at a day length of 16 hours (120 µmol/m^2s), air humidity of 60 % and a temperature of 22 °C. *C. sativa* plants were grown non-sterile on soil (Frühstorfer Erde Typ T 25, Industrie Erdwerk Archut, Lauterbach-Wallenrod, Germany) in the greenhouse at a day length of 16 hours and temperatures between 16-22 °C. The herbicide Basta (Bayer CropScience, Monheim, Germany) containing glufosinate as active ingredient was used for selection of transgenic plants and sprayed one and two weeks after sowing onto the plants.

2.11 Molecular cloning methods

2.11.1 Isolation of RNA from algae cultures or plant material

Total ribonucleic acid (RNA) was isolated using the protocol described by (Vicient and Delseny, 1999). Prior to usage, solutions for RNA isolation were treated with 0.1 % (v/v) diethylpyrocarbonate (DEPC) for at least 1 hour at 37 °C to inactivate RNAses. Then, solutions were autoclaved for 20 min at 120 °C to destroy residual DEPC. Algal material was harvested by centrifugation at 4 °C and 3 320 *relative centrifugal force* (*x g)* for 10 min. Pellets were transferred into sterile 2 ml-tubes and frozen in liquid nitrogen. Afterwards, about 500 µl glass beads and 2 ml cold extraction buffer (8 M LiCl, 2 % (v/v) β-mercaptoethanol) were added. Plant material was ground in liquid nitrogen and 2 ml cold extraction buffer were added. Samples were vortexed and incubated overnight at 4 °C. On the next day, samples were centrifuged for 4 seconds (s) to remove cell debris. Supernatants were placed into fresh 1.5 ml-tubes and centrifuged at 18 000 *x g* for 30 min at 4 °C. Supernatants were discarded and pellets were washed with cold 70 % (v/v) ethanol. Afterwards, they were dissolved in 0.5 ml solubilization buffer (0.5 % (w/v) sodium dodecyl sulphate (SDS), 100 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 10 mM trishydroxymethylaminomethane (Tris)-

HCl, pH 7.6). The aqueous phase was extracted once with an equal volume of phenol (pH 7.6), once in phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and once in chloroform/isoamyl alcohol (24:1, v/v). The aqueous phase was transferred into fresh 1.5 ml-tubes and 0.1 volume 3 M NaOAc solution as well as 1.5 volume ethanol were added. Samples were incubated for 2 hours at -80 °C and then centrifuged for 30 min at 4 °C and maximum speed. Supernatants were discarded and 0.5 ml 3 M NaOAc solution was added to the pellets. Samples were vortexed for 1 min and then centrifuged for 10 min at 4 °C and maximum speed. Supernatants were discarded again. Pellets were washed with 70 % (v/v) ethanol, dried at 23 °C and then dissolved in 20 µl ddH_2O.

2.11.2 cDNA synthesis

Copy deoxyribonucleic acid (cDNA) can be synthesized from messengerRNA (mRNA) by reverse transcription (Mullis and Faloona, 1987). For this method, isolated template mRNAs as well as RNA-dependent deoxyribonucleic acid (DNA) polymerase, deoxyribonucleotides (dNTPs) and oligo$(dT)_{18}$-primers are required. Prior to the reverse transcription process, the isolated RNA was treated with DNAseI (Fermentas, St. Leon Rot, Germany) according to the manufacturer's instructions to degrade residual genomic DNA in the sample. Afterwards, RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon Rot, Germany) was used to prepare cDNA according to the given recommendations in the manual.

2.11.3 Isolation of genomic DNA from yeast pellets and plant material

Genomic DNA was isolated with cetyltrimethylammoniumbromid (CTAB) to verify genomic mutations in knockout yeast as well as in mutant plants. Yeast material was centrifuged at 4 °C and 3 320 *x g* for 10 min, cell pellets were transferred into 1.5 ml-tubes and frozen in liquid nitrogen. Plant tissue was pulverized in 1.5 ml-tubes using plastic pestles and liquid nitrogen. 250 µl CTAB solution (2 % (w/v) CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl) containing 2 % (v/v) β-mercaptoethanol were added to the samples. These were incubated for 30 min to 1 hour at 65 °C. Afterwards, an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and samples were mixed well. Tubes were centrifuged at 7 500 *x g* for 3 min at 23 °C. 200 µl of the upper aqueous phase were transferred into fresh tubes, and 1/10 volume CTAB/NaCl solution (10 % (w/v) CTAB, 0.7 % (w/v) NaCl; preheated to 65 °C) was added. Samples were mixed well and incubated for 2 min at 23 °C. Afterwards, an equal volume of isopropanol was added, samples were mixed by inverting the tubes several times and again incubated for 2 min at 23 °C. Tubes were centrifuged at 20 000 *x g* and 4 °C for 10 min. Supernatants were discarded and pellets were washed with 100 µl 75 % (v/v) ethanol. The ethanol was removed, pellets were dried at 23 °C and then dissolved in 70 µl ddH_2O.

2.11.4 Separation of DNA and RNA in agarose gels

DNA and RNA fragments of different sizes were separated via electrophoresis in gels prepared from 1-2 % (w/v) agarose in TAE buffer (40 mM Tris-acetate, pH 8.3, 2 mM EDTA). Prior to loading RNA samples, gel chamber, sliding carriage and gel comb were incubated for at least 20 min in 1 % (w/v) SDS solution to inactivate RNAses. Before loading the samples into the gel pockets they were mixed with loading buffer (40 % (v/v) glycerol, 100 mM EDTA, pH 8.0, 0.1 % (w/v) SDS, 0.25 % (w/v) bromphenolblue, 0.25 % (w/v) xylencyanol) in the ratio 10:1. For length and concentration determination of the loaded DNA fragments 2.5 µg GeneRuler 1 kb DNA Ladder (Fermentas, St. Leon Rot, Germany) were loaded into a separate gel pocket. Gels were run in TAE buffer for 30 min at 13 V/cm. Afterwards, gels were incubated for 15 min in an ethidium bromide bath (2 µg/ml ethidium bromide in TAE buffer), afterwards DNA bands were visualized with the gel detection system IDA (Raytest, Straubenhardt, Germany).

2.11.5 Polymerase chain reaction (PCR)

PCR is used to amplify a specific DNA sequence from a given template, like genomic DNA, cDNA or plasmid DNA. Besides the template, also dNTPs, primers binding to the 3'- and 5'-ends of the selected DNA sequence as well as thermostable DNA-dependent DNA polymerase are required for this method. The PCR is separated into the three different phases of denaturation, annealing and extension. During denaturation, the DNA template is split into its two complementary strands. During annealing, primers are binding to their complementary sequences and can then be used during extension as starting points for the polymerase to synthesize the complete complementary strand. This cycle is repeated 25-35 times, resulting in an exponential amplification of the desired sequence in the reaction mixture. The PCR reactions described in this work were performed with Advantage PCR Enzyme System (Clontech, Mountain View, CA, USA), TaKaRa ExTaq DNA polymerase (Cambrex BioScience, Potsdam, Germany) or Phusion DNA polymerase (Finnzymes, Espoo, Finland). For analytical purposes 25 µl-reactions and for preparative purposes 50 µl-reactions were set up according to the manufacturer's recommendations. A typical PCR profile for Phusion DNA polymerase is given below and was used, if not stated otherwise.

1. 98 °C 1 min
2. 98 °C 30 s
3. T_m-3 °C 30 s
4. 72 °C 30 s/kb

   Steps 2-4 are repeated for 30 times
5. 72 °C 3 min

T_m is the specific melting temperature of the chosen primers and can be calculated as follows (Suggs et al., 1981):

T_m [°C] = 2*(sum of A + T) + 4*(sum of G + C)

Primers were ordered from Sigma (Munich, Germany) or Invitrogen (Karlsruhe, Germany), primer sequences of utilized primers are listed in the appendix.

2.11.6 Restriction digest

Restriction enzymes cut specifically in palindromic DNA sequences, thereby either leading to sticky or blunt ends that can subsequently be ligated with ends of the same kind and thus are helpful tools for molecular cloning. Plasmid DNA or PCR-derived DNA fragments were treated with one to two restriction enzymes according to the manufacturer's instructions. This method was used to either verify the presence of a DNA fragment after plasmid DNA isolation or to prepare both insert and vector for (sub-)cloning procedures. For the former purpose a 3 µl-aliquot of isolated plasmid DNA was treated with restriction enzymes and then run on an agarose gel (see 2.11.4) to check for the right fragment size. For the latter purpose both vectors, the donor vector containing the DNA fragment of interest and the acceptor vector for this fragment, were cut with the same combination of restriction enzymes. The restriction digest reaction of the acceptor vector was stopped either by incubation at 85 °C for 20 min or by gel-purification (see 2.11.7). In some cases, the acceptor vector was also treated with Calf Intestine Alkaline Phosphatase (Fermentas, St. Leon Rot, Germany) according to manufacturer's instructions to prevent single-cut vectors from religation later on. The insert fragment was gel-purified to remove donor vector and restriction enzymes prior to ligation.

2.11.7 Isolation of DNA fragments from agarose gels

DNA fragments were isolated from agarose gels by running them in the gel as described in section 2.11.4. The respective DNA band was then cut out of the gel. Afterwards, NucleoSpin Extract Kit (Macherey-Nagel, Düren, Germany) was used for eluting the DNA according to the manufacturer's protocol.

2.11.8 Ligation

Gel-purified PCR fragments were ligated with the TA cloning vector pGEM-T (Promega, Madison, WI, USA) according to the manufacturer's proctocol. When the preceding PCR had been performed with Phusion DNA polymerase (Finnzymes, Espoo, Finland), DNA fragments had to be treated with TaKaRa ExTaq DNA polymerase prior to ligation in order to add a poly-A overhang. In those cases, a mix containing the PCR reaction, 1 µl 100 mM dNTPs and 0.1 µl TaKaRa ExTaq DNA polymerase was incubated for 10 min at 72 °C. DNA fragments after restriction digest were ligated with yeast or plant expression vectors, treated as described in section 2.11.6, in the ratio 3:1 using 5 units T4 DNA ligase according to the manufacturer's manual (Fermentas, St. Leon Rot, Germany). Ligation reactions were then incubated overnight at 4 °C.

2.11.9 Gateway Cloning

An alternative way to combine different DNA fragments without using restriction enzymes and ligation procedures is the application of clonase enzyme. This enzyme specifically recombines sequences on donor and acceptor plasmids and thereby transfers DNA fragments from one vector to the other. For cloning, Gateway LR Clonase II Enzyme Mix (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instructions.

2.11.10 Transformation of *E. coli*

By the method of transformation, free DNA can be introduced into an acceptor organism, thereby changing its genetic composition. By culturing transformed bacteria, the transferred DNA construct can for example be amplified for subsequent isolation. Many bacteria have to be treated beforehand with chemicals like calcium chloride to enhance their ability to take up foreign DNA.

2.11.10.1 Preparation of chemically competent *E. coli* cells

Competent cells were prepared according to (Inoue et al., 1990). For preculture 5 ml LB medium containing tetracycline (10 mg/l) were set up overnight with *E. coli* XL1blue cells at 37 °C while shaking at 200 rpm. On the next day, 400 ml LB medium with tetracycline were inoculated with the whole preculture and cells were grown at 37 °C while shaking at 200 rpm until they reached an optical density at 600 nm (OD_{600}) of 0.6. Bacteria were incubated on ice for 10 min, afterwards centrifuged for 10 min at 4 °C and 1 000 *x g*. The medium was completely removed, cells were resuspended in 120 ml transformation buffer (TFB; 10 mM piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes), 15 mM $CaCl_2$, 250 mM KCl and 55 mM $MnCl_2$) and incubated on ice for 10 min. Then they were centrifuged at 4 °C and 1 000 *x g*. Buffer was removed completely, cells were resuspended in 32 ml TFB buffer containing 7 % (v/v) DMSO and incubated again for 10 min on ice. Afterwards, they were aliquoted into units of 200-500 µl, frozen in liquid nitrogen and stored at -80 °C until use.

2.11.10.2 Transformation of competent *E. coli* cells

For transformation, competent cells were thawn gently on ice. Then, 100 µl of cells were added either to 10 µl ligation reaction, 5 µl clonase reaction mix or 1 µl plasmid DNA and incubated on ice for 20 min. Cells were put to 42 °C for 35 s and then immediately placed on ice again. 600 µl LB medium were added to the cells and they were incubated for one hour at 37 °C while shaking at 200 rpm. Then, the appropriate amount of cells was plated on solid LB medium containing the respective antibiotic as selection marker. Plates were incubated overnight at 37 °C.

2.11.11 Isolation of plasmid DNA from *E. coli*

For plasmid DNA isolation, positive clones were chosen after transformation and inoculated overnight at 37 °C while shaking at 200 rpm in 2 ml liquid LB medium containing the respective antibiotic as selection marker. NucleoSpin Plasmid Kit from Macherey-Nagel (Düren, Germany) was then used according to the manufacturer's instructions.

2.11.12 Sequencing

Sequencing is crucial to prove the accuracy of PCR-amplified sequences in plasmids. The method modified from (Sanger et al., 1977) is based on intermixed dideoxynucleotides (ddNTPs) in the PCR reaction which lead to abortion of chain elongation. They are conjugated with base-specific fluorescent dyes, thereby the sequence of the analyzed DNA fragment can be read out. For sequencing, ABI Prism BigDye Terminator (BDT) Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Foster, USA) and sequence- as well as vector-specific primers were used. 200-300 ng plasmid DNA as template, 1.5 µl BDT Ready Reaction Mix, 1.5 µl half term buffer, 1 µl sequencing primer (0.5 µM) and 5 µl sterile ddH_2O were mixed in PCR tubes. PCR parameters were initial denaturation for 1 min at 96 °C followed by 25 cycles 96 °C denaturation for 5 s, 55 °C annealing for 15 s and 60 °C extension for 4 min. After PCR, reactions were transferred into fresh 1.5 ml-tubes and DNA was precipitated by mixing samples with 1 µl 3 M NaOAc (pH 5), 1 µl 125 mM EDTA and 25 µl 96 % (v/v) ethanol. Samples were incubated for 15 min at 23 °C and then centrifuged at maximum speed for 15 min in a microcentrifuge. Supernatants were discarded and DNA pellets were washed with 50 µl 70 % (v/v) ethanol. Supernatants were discarded and pellets were dried for 1 min at 90 °C. Then they were resuspended in 15 µl formamide and stored at -20 °C until analysis. This was performed with ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster, USA). Evaluation of the obtained data was done using the program Chromas lite (www.technelysium.com.au) as well as the online softwares MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html, according to (Corpet, 1988) and Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

2.12 Isolation and cloning of studied cDNA sequences

2.12.1 Cloning of microalgal acyltransferase sequences into yeast expression vectors

OlLPCAT from *O. lucimarinus* was ordered as completely codon-optimized version by Dr. M. Wagner. *OtLPCAT* had been isolated from *O. tauri* and partly codon-optimized by Dr. M. Heilmann. Templates for the conducted PCRs are listed in section 2.6. *OlLPCAT* was amplified using primers *OlLPCATcoforBamHI* and *OlLPCATcorevApaI* (all primers are listed in the appendix). *OtLPCAT* was amplified using primers *OtLPCATtcoforBamHI* and *OtLPCATtcorevNheI*. Resulting DNA fragments containing a translation initiation signal were treated with the respective restriction enzymes, gel-

purified and ligated into pESC-LEU cut with the same combinations of enzymes. *OtLPCAT* in addition was also cloned into pYES2/CT. For creating a nucleotide sequence without stop codon, primer combination *OtLPCATtcoforBamHI* and *OtLPCATtcorev-StopXbaI* was used. In order to obtain a PCR-derived sequence with stop codon, primers *OtLPCATtcoforBamHI* and *OtLPCATtcorevNotI* were utilized. Resulting DNA fragments containing a translation initiation signal were treated with the respective restriction enzymes, gel-purified and ligated into pYES2/CT cut with the same combinations of enzymes. Positive clones were sequenced and then used for transformation of yeast.

To generate GFP-fusion constructs, appropriate restriction sites were added by PCR using primers *OlLPCATcoforHindIII* and *OlLPCATcorevXhoI* or *OtLPCATtcoforSpeI* and *OtLPCATtcorevHindIII*, respectively. The resulting PCR products were gel-purified, ligated into pUG36 cut with the same combinations of enzymes and sequenced prior to transformation into yeast.

OtDGAT2A and *OtDGAT2C* were ordered as completely codon-optimized versions by Dr. M. Wagner. Templates for the conducted PCRs are listed in section 2.6. *OtDGAT2A* and *OtDGAT2C* were amplified using primers *OtDGAT2AcoforEcoRI* and *OtDGAT2AcorevNotI* or *OtDGAT2CcoforEcoRI* and *OtDGAT2CcorevNotI*, respectively. Resulting DNA fragments containing a translation initiation signal were treated with the corresponding restriction enzymes, gel-purified and ligated into mCherryFAR1-pESC-URA (provided by Dr. M. Heilmann) cut with the same combination of enzymes. Positive clones were sequenced and then used for transformation of yeast.

2.12.2 Isolation, codon-optimization and cloning of *Old4* from *O. lucimarinus* into different yeast expression vectors

Old4 was isolated from genomic DNA (provided by Dr. M. Heilmann) using primers *Old4for*, *Old4hppgfor*, *Old4mfor*, *Old4hppgrev*, *Old4mrev* and *Old4rev*. The front and middle fragments were amplified by PCR using the Advantage PCR Enzyme System and primer combinations *Old4for-Old4hppgrev* and *Old4hppgfor-Old4mrev*, respectively. PCR parameters were initial denaturation for 3 min at 95 °C followed by 25 cycles 95 °C denaturation for 30 s, 70 °C annealing for 30 s and 72 °C extension for 90 s. In the end, a final 72 °C extension phase of 5 min was added. The rear fragment of the gene was amplified with Phusion DNA polymerase. The primers used were *Old4mfor* and *Old4rev*. PCR parameters were initial denaturation for 3 min at 98 °C followed by 25 cycles 98 °C denaturation for 30 s, 60 °C annealing for 30 s and 72 °C extension for 70 s. In the end, a final 72 °C extension phase of 5 min was added. Afterwards, an overlap extension PCR with Phusion DNA polymerase using primers *Old4for* and *Old4rev* and the three different fragments as template was performed. PCR parameters were initial denaturation for 2 min at 98 °C followed by 10 cycles 98 °C denaturation for 15 s, 68 °C annealing for 30 s and 72 °C extension for 60 s. Then, 20 cycles of 98 °C denaturation for 15 s, 70 °C annealing for 30 s and 72 °C extension for 60 s followed. In the end a final 72 °C extension phase of 7 min was added.

The resulting DNA fragment was gel-purified and cloned into pGEM-T for sequencing. Phusion DNA polymerase was used for the following PCR reactions using a positive clone as template. Codon-optimization of the first 20 codons for the expression in yeast was performed using primers *Old4tcofor* and *Old4rev*. In a second reaction, restriction sites and an *ACATA* nucleotide sequence as translation initiation signal in front of the start codon (Donahue and Cigan, 1990) were introduced into the sequence using primers *Old4tcoforEcoRI* and *Old4revNotI*. PCR parameters were initial denaturation for 1 min at 98 °C followed by 25 cycles 98 °C denaturation for 30 s, 50-60 °C annealing for 1 min and 72 °C extension for 70 s. In the end, a final 72 °C extension phase of 5 min was added. All PCR reactions of this type were performed in the Mastercycler gradient Eppendorf AG (Hamburg, Germany). The resulting PCR product was cloned into the *EcoRI* and *NotI* sites of the vector pESC-TRP under the control of the inducible promoter *GAL10*. The resulting construct was sequenced before transformation into yeast. To generate a GFP-fusion construct, the prepared pESC-TRP construct was used as template for amplification and addition of appropriate restriction sites by PCR using primers *Old4tcoforEcoRI* and *Old4revXhoI*. The resulting PCR product was gel-purified, ligated into the *EcoRI* and *XhoI* sites of pUG36 and sequenced prior to transformation into yeast.

2.12.3 Cloning of *Egd4* from *E. gracilis* into different yeast expression vectors

The construct MsΔ6-PSE1-MsΔ5-OtELO5-Egd4-pCAMBIA3300 was provided by S. Schlenczek. The open reading frame of *Egd4* was amplified using primers *Egd4forBamHI* and *Egd4revXhoI* in a “touch-down” PCR using Phusion DNA polymerase. PCR parameters were initial denaturation for 2 min at 98 °C followed by 20 cycles 98 °C denaturation for 20 s, 72 °C annealing for 30 s and 72 °C extension for 70 s. During each cycle, the annealing temperature was lowered 0.1 °C/s. Then, 20 cycles 98 °C denaturation for 20 s, 70 °C annealing for 30 s and 72 °C extension for 70 s followed. During each cycle, the annealing temperature was lowered 0.3 °C/s. The resulting DNA fragment was sublconed into pGEM-T. A positive clone was subjected to sequencing and then used for restriction digest with *BamHI* and *XhoI*. The resulting fragment was cloned into the *BamHI* and *XhoI* sites of pESC-TRP. To generate a GFP-fusion construct, the prepared pESC-TRP construct was used as template for amplification and addition of appropriate restriction sites by PCR using primers *Egd4forEcoRI* and *Egd4revXhoI*. The resulting PCR product was cloned into the *EcoRI* and *XhoI* sites of pUG36 and sequenced prior to transformation into yeast.

2.12.4 Isolation and cloning of *TpLACS* from *T. pseudonana* into a yeast expression vector

The long-chain acyl-CoA synthetase nucleotide sequence from *T. pseudonana* (Tonon et al., 2005a) was isolated via PCR from cDNA prepared from total RNA using primers *TpLACSfor* and *TpLACSrev*. The resulting PCR product was used as template in a second PCR reaction to add restriction sites and a translation initiation signal with primers *TpLACSforApaI* and *TpLACSrevNheI*. The resulting DNA fragment was subcloned into pGEM-T. A positive clone was subjected to sequencing and then used for restriction digest with *ApaI* and *NheI*. The resulting fragment was cloned into the *ApaI* and *NheI* sites of pESC-LEU.

2.12.5 Isolation and cloning of *CsMGDGS* from *Cucumis sativus* into a yeast expression vector

The monogalactosyldiacylglycerol synthase nucleotide sequence from *C. sativus* (Shimojima et al., 1997) was amplified using a cDNA bank prepared from four days old etiolated cucumber seedlings (provided by Dr. E. Hornung) as template. The sequence was amplified without its chloroplastidial targeting signal utilizing primers *CsMGDGSforBamHI* and *CsMGDGSrevNheI* in a “touch-down” PCR as described in section 2.12.3. The resulting PCR product containing a translation initiation signal was cloned into the *BamHI* and *Nhe*I sites of pESC-LEU.

2.12.6 Cloning of microalgal and plant acyltransferase sequences into plant expression vectors

OtDGAT2B, *OlLPCAT* and *OtPDAT* were isolated by Dr. M. Wagner from *O. tauri* and *O. lucimarinus*, respectively. *OtLPCAT* was isolated from *O. tauri* and *AtDGAT1* as well as *AtDGAT2* were isolated from *A. thaliana* by Dr. M. Heilmann. Templates for the conducted PCRs are listed in section 2.6. *OtDGAT2B* was amplified using primers *OtDGAT2BtcoforBamHI* and *OtDGAT2BtcorevNotI*. *OlLPCAT* was amplified using primers *OlLPCATcoforBamHI* and *OlLPCATcorevNotI*. *OtPDAT* was amplified using primers *OtPDATtcoforEcoRI* and *OtPDATtcorevNotI*. *OtLPCAT* was amplified using primers *OtLPCATtcoforBamHI* and *OtLPCATtcorevNotI*. *AtDGAT1* was amplified using primers *AtDGAT1forKpnI* and *AtDGAT1revXhoI*. *AtDGAT2* was amplified from the template using primers *AtDGAT2forEcoRI* and *AtDGAT2revXhoI*. For all DNA fragments, “touch-down” PCRs like described in section 2.12.3 were performed. Afterwards, they were treated with the respective restriction enzymes and ligated into the pUC18-Entry2 cut with the same combinations of enzymes. Positive clones were sequenced and subjected to clonase reaction (see section 2.11.9) with pCAMBIA33.2cGs. Clonase reaction mixtures were used for *E. coli* transformation.

2.12.7 Cloning of *Msd6* and *OlLPCAT* into Ptd6-PSE1-Ptd5-pCAMBIA3300

Ptd6-PSE1-Ptd5 and *Msd6* or *Msd6-OlLPCAT* or *OlLPCAT*, respectively, were inserted sequentially into pCAMBIA3300 by first digesting the destination vector with *SbfI* and ligating it with the expression cassette cut out from Ptd6-PSE1-Ptd5-pUC19-USP-OCS1 with *SbfI*. A positive clone was then cleaved with *AscI* and ligated with the expression cassettes cut out from Msd6-pUC19-USP-OCS123 or Msd6-OlLPCAT-pUC19-USP-OCS123 or OlLPCAT-pUC19-USP-OCS123 with *AscI*. Positive clones were used for transformation of *A. tumefaciens*.

2.13 Genetic engineering methods

2.13.1 Transformation of *A. tumefaciens*

A. tumefaciens cells were transformed with plasmid DNA isolated from *E. coli* in order to use the positive transformants for subsequent *A. tumefaciens*-mediated transformation of *A. thaliana* or *C. sativa* plants.

2.13.1.1 Preparation of chemically competent *A. tumefaciens* cells

For preparation of chemically competent cells, the method developed by (Höfgen and Willmitzer, 1988) was applied. For preculture, 2 ml YEB medium containing rifampicin (50 mg/l) were set up overnight with *A. tumefaciens* cells at 28 °C while shaking at 200 rpm. On the next day, 50 ml YEB medium were inoculated with the whole preculture and cells were grown at 28 °C while shaking at 200 rpm for 4 hours until they reached an OD_{600} of 0.5. Bacteria were centrifuged at 4 °C and 2 300 *x g* for 5 min. The medium was completely removed and cells were resuspended in 10 ml 0.15 M NaCl solution. Then they were centrifuged again, the buffer was completely removed and cells were resuspended in 1 ml ice cold 75 mM CaCl solution. Afterwards, 200 µl-aliquots were taken, frozen in liquid nitrogen and stored at -80 °C until use.

2.13.1.2 Transformation of competent *A. tumefaciens* cells

For transformation, 200 µl-aliquots with competent cells were thawn at 37 °C. Then, 3 µg plasmid DNA were added and cells were incubated at least for 30 min on ice. Once in a while they were mixed gently. Afterwards, the mix was frozen for 2 min at -80 °C and immediately thawn again at 37 °C. 800 µl YEB medium were added to the cells and they were incubated for 1-4 hours at 28 °C while shaking at 200 rpm. Then, cells were sedimented, plated on solid YEB medium containing the respective antibiotic as selection marker and incubated for 2 days at 28 °C.

2.13.2 Transformation of *S. cerevisiae*

S. cerevisiae cells were transformed according to (Ito et al., 1983) with plasmid DNA isolated from *E. coli* or with linear fragments produced by PCR. Plasmid DNA was transformed in order to use the positive yeast transformants for subsequent biochemical analysis of the encoded enzymes. Selection was based on the auxotrophy of the utilized yeast strains for several amino acids. The auxotrophic marker was combined with the cDNA of interest on a yeast vector. Alternatively, linear fragments were transformed to obtain knockout mutants. Then, selection was based on the acquired resistance of the knockout yeast towards hygromycin B as described in section 2.13.3.

As preculture, 2 ml YPD medium were inoculated overnight with the respective yeast strain and shaken at 30 °C and 200 rpm. On the next day, 5 ml YPD medium per transformation reaction were inoculated with the appropriate amount of preculture to have a starting OD_{600} of 0.2. Cells were grown at 30 °C while shaking at 200 rpm until their OD_{600} reached 0.8. Cells were centrifuged at 1 600 *x g* for 3 min at 23 °C, the supernatant was discarded and cells were washed once with 5 ml sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Cells were resuspended in 100 µl 0.1 M lithium acetate in TE buffer and incubated for 10 min at 23 °C. The suspension was mixed with either 5 µl plasmid DNA or 50 µl purified PCR product combined with 10-25 µl denatured single stranded herring sperm carrier DNA and 700 µl transformation mix (40 % (w/v) polyethylene glycol (PEG) 4000, 0.1 M lithium acetate, 10 mM Tris-HCl, 1 mM EDTA, pH 8). Suspensions were incubated for 30 min at 30 °C while shaking at 200 rpm. Afterwards, they were shifted to 42 °C for 15 min and centrifuged at 4 500 *x g* for one minute. Supernatants were removed, cells transformed with plasmid DNA were washed in 500 µl TE buffer and then resuspended in 50 µl TE buffer. Afterwards, they were plated on solid SD medium containing 2 % (w/v) glucose and the respective 50 x synthetic complete drop-out medium mix for selection of the transformants. In case of transformation with linear DNA for gene deletion, cells were resuspended in 1 ml YPD medium and incubated for another 2-3 hours at 30 °C while shaking at 200 rpm. Then, cells were sedimented and plated on solid YPD medium containing hygromycin B for selection purposes.

2.13.3 PCR-based gene deletion in *S. cerevisiae*

In order to obtain different yeast knockout strains, PCR-based gene deletion according to (Wach et al., 1994; Guldener et al., 1996; Goldstein and McCusker, 1999) was applied. This technique is based on the fact that homologous recombination with linear DNA fragments occurs very easily in yeast. Linear DNA fragments for knockout creation were prepared by fusing a hygromycin B phosphotransferase gene containing *hphMX4*-resistance cassette with flanking 3'- and 5'-sequences of the gene that had to be removed in a PCR reaction. As template for the PCR, plasmid DNA containing the resistance cassette was used, and for every gene specific primers were designed (pro-

vided by Dr. Martin Fulda and Dr. Michael Scharnewski). The PCR product was purified using NucleoSpin Extract Kit (Macherey-Nagel, Düren, Germany) and transformed into yeast (see 2.13.2). During transformation, the gene of interest was then replaced by homologous recombination with the introduced PCR product. Positive transformants were selected on YPD medium containing hygromycin B. Genomic DNA was isolated from these clones (see 2.11.3) and used as template for control PCRs checking for successful knockout of the gene of interest. For these PCRs, gene specific as well as resistance cassette specific primers were used.

2.13.4 Transformation of plants

Flourishing *A. thaliana* and *C. sativa* plants were transformed by the flower-dipping method developed by (Clough and Bent, 1998) using *A. tumefaciens* EHA105 cells as mediating vector. Transgenic T1 plants were then selected by kanamycin and/or Basta in order to obtain T2 transgenic seeds for biochemical analyses or sowing to gain T3 seeds.

2.13.4.1 Transformation of *A. thaliana*

Transformed *A. tumefaciens* cells were cultivated in 400 ml YEB medium containing the required selection antibiotics at 28 °C while shaking at 200 rpm until they reached an OD_{600} of 2. Afterwards, cells were centrifuged at 4 °C and 2 000 *x g* for 10 min and resuspended in 200 ml 5 % (w/v) sucrose-solution. Suspensions were incubated for 20 min on ice, afterwards 100 µl 0.05 % (v/v) Silwet L-77 (OSI Specialties, Danbury, CT, USA) were added to reduce the surface tension of the flowers. The influorescences of *Arabidopsis* plants were dipped for approximately 5 s into the bacterial solution. After dipping, plants were kept for at least 4 hours out of direct light and under a plastic cover in order to assure high air humidity, before they were put back to the greenhouse.

2.13.4.2 Transformation of *C. sativa*

The method applied was similar to the one established by (Lu and Kang, 2008) and included vacuum infiltration. Bacterial cells were prepared as described above (see 2.13.4.1). After incubation on ice, the beaker with the cell suspension was placed in an exsiccator. Up to six *Camelina*-plants were positioned around the beaker, their influorescences were dipped into the bacterial solution. The exsiccator was closed and a vacuum of -40 kPa was generated. It was held for 5 min, then air was allowed to stream into the exsiccator in a slow and controlled way. After dipping, plants were also kept for at least 4 hours out of direct light and under a plastic cover, before they were put back to the greenhouse.

2.14 Heterologous expression of cDNAs in *S. cerevisiae*

For functional characterization of enzymes, the respective cDNAs were expressed in different yeast strains that were auxotrophic for several amino acids. This feature was used for selection of transgenic cells containing the desired cDNA sequence combined with an auxotrophic marker on a yeast vector. In vectors containing the *GAL1* and/or the *GAL10* promoter like pESC- and pYES-vectors, expression was induced by galactose. Expression using the vector pUG36 was induced by absence of methionine.

As precultures, 2-10 ml SD medium containing 2 % (w/v) raffinose and the respective synthetic dropout mix for selection were inoculated with transformed yeast colonies. Cultures were grown overnight at 30 °C while shaking at 200 rpm. On the next day 10-100 ml SD medium containing 2 % (w/v) galactose for expression induction and the respective amino acid dropout mix were inoculated with the appropriate amount of preculture to have a starting OD_{600} of 0.3. In case substrate specificity of enzymes should be investigated, also 1 % (v/v) Igepal CA 630 (Fluka, Steinheim, Deutschland) and 200 µM of free fatty acid were added. Igepal is a detergent and permeabilizes cell membranes to enhance the uptake of very long chain polyunsaturated and thus unpolar fatty acids. For protein expression tests, cultures were grown for 20-48 hours at 23 °C while shaking at 150 rpm. In case of methionine-dependent expression, cultures were cultivated either in presence of 0.3 mM methionine (moderate expression) or in complete absence of methionine (over-expression). For protein activity tests with or without substrates, cultures were grown for 24-96 hours at temperatures between 16-30 °C while shaking at 150-200 rpm, as indicated for each experiment. Afterwards, OD_{600} of the cultures was determined and cells were harvested by centrifugation for 5 min at 1 500 *x g*. Supernatants were discarded and cells were washed twice with 5 ml sterile ddH_2O. The resulting cell pellet was then used for protein or lipid analyses.

2.15 Microscopic procedures

Yeast cells expressing GFP-Old4-pUG36 were grown in 2 ml-cultures at 30 °C to stationary phase while shaking at 200 rpm. For staining of nuclei, cells were incubated for 15 min with the dye Hoechst 33342 trihydrochloride (Molecular Probes, Invitrogen, Karlsruhe, Germany). Fluorescence and light microscopy was done using a Zeiss Axioscope2 microscope and an Axiocam digital camera. Pictures are pseudo-colorized.

2.16 Protein expression analysis

2.16.1 Protein sample preparation

Yeast cultures for recombinant protein expression tests were grown as described above. The sample preparation was then performed according to pYES2/CT manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). Cell pellets from 20 ml-cultures were resuspended in 500 µl sterile ddH_2O and transferred to sterile 2 ml-tubes. Samples were centrifuged for 30 s at maximum speed, supernatants were removed and pellets were either stored at -80 °C or directly used to prepare cell lysates. For this purpose, cells were resuspended in 500 µl of breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5 % (v/v) glycerol, 1 mM phenylmethanesulphonylfluoride (PMSF)) and centrifuged at 1 500 *x g* for 5 min at 4 °C. Supernatants were removed and cells were resuspended in 200 µl breaking buffer. An equal volume of glass beads (1.7-2 mm; Carl Roth & Co., Karlsruhe, Germany) was added and samples were vortexed eight times for 30 s, each time followed by 30 s on ice. Then they were centrifuged for 10 min at maximum speed in a microcentrifuge, supernatants were transferred to fresh tubes and the lysates were assayed for protein concentration using Bradford solution (0.007 % (w/v) Coomassie Brilliant Blue G-250, 4.8 % (v/v) ethanol, 8.5 % (v/v) phosphoric acid) and bovine serum albumin (BSA) as a standard according to (Bradford, 1976). The dye is binding the proteins in the sample and thereby changes its colour which can be measured with a spectrophotometer. For determination of protein concentration 2 ml Bradford reagent were mixed with 2 µl of sample and 98 µl ddH_2O. The mixture was incubated for 5 min at 23 °C and then the absorption was measured at λ = 595 nm.

2.16.2 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was performed to separate proteins of different molecular weights on the basis of (Laemmli, 1970) using Mini-PROTEAN 3 System (Biorad, Hercules, CA, USA), a 5 % stacking gel and a 12 % separation gel according to manufacturer's instructions. For protein size prediction, 5 µl of Prestained Protein Molecular Weight Marker (Fermentas, St. Leon Rot, Germany) were loaded. 5 x SDS-PAGE sample buffer (0.225 M Tris-HCl, pH 6.8, 50 % (v/v) glycerol, 5 % (w/v) SDS, 0.05 % (w/v) bromphenolblue, 0.25 M dithiothreitol) was added to a final concentration of 1 x in order to introduce an excess of negative charge into the proteins contained in the samples. Then, samples were boiled for 5 min to denature the proteins. 20 µg of lysate were then used for SDS-PAGE electrophoresis. Protein gels were run at 200 V in running buffer (25 mM Tris-HCl, pH 8.9, 0.2 M glycine, 0.1 % (w/v) SDS). After the run, gels were either stained with Coomassie Brilliant Blue G-250 or used for Western Blotting.

Stacking gel (5 %)

59 % (v/v)	ddH_2O
23 % (v/v)	Gel buffer (3 M Tris-HCl, pH 8.45, 0.3 % (w/v) SDS)
18 % (v/v)	Acrylamide solution (30 %, containing 0.8 % bisacrylamide)
0.4 % (v/v)	Tetramethylethylenediamine (TEMED)
0.04 % (w/v)	Ammonium persulfate (APS)

Separation gel (12 %)

40 % (v/v)	Acrylamide solution (30 %, containing 0.8 % bisacrylamide)
33 % (v/v)	Gel buffer (3 M Tris-HCl, pH 8.45, 0.3 % (w/v) SDS)
27 % (v/v)	ddH_2O
0.1 % (v/v)	TEMED
0.05 % (w/v)	APS

2.16.3 Coomassie staining

For visualization of proteins, gels were stained for 30 min in Coomassie staining solution (40 % (v/v) methanol, 10 % (v/v) acetic acid, 0.25 % (w/v) Coomassie Brilliant Blue G-250) on the basis of (Meyer and Lamberts, 1965). Acetic acid fixes the proteins in the gel, the dye binds basic and aromatic amino acids and thereby unspecifically stains all proteins in the gel. Unbound dye was removed by shaking gels in ddH_2O for several hours.

2.16.4 Western Blot analysis and immunodetection

For immunodetection of epitope-tagged proteins, these were transferred from the SDS-PAGE gel to nitrocellulose membrane (Macherey&Nagel, Düren, Germany) using Mini Trans-Blot cell equipment (Bio-Rad, Hercules, CA, USA). Transfer was performed for 90 min at 60 V in the presence of prechilled transfer buffer (0.05 M Tris-HCl, 0.04 M glycine, 20 % (v/v) methanol, 0.4 % (w/v) SDS). Following transfer, the nitrocellulose membrane was washed in TBST buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 % (v/v) Tween20) and incubated with blocking solution (5 % (w/v) BSA in TBST buffer) overnight at 4 °C while shaking on a rocking table. This procedure was performed to minimize unspecific binding of the antibodies. On the next day, the membrane was washed three times for 15 min while shaking in TBST buffer to remove the blocking solution. In order to detect recombinant proteins, the membrane was incubated for 2-16 hours at 23 °C with the primary antibody diluted according to the manufacturer's instructions in TBST buffer containing 5 % (w/v) BSA on the rocking table. After washing the membrane three times with TBST buffer for 15 min the secondary antibody di-

luted in TBST buffer according to the manufacturer's instructions was added and incubated for 1 hour at 23 °C while shaking. The membrane was washed three times for 15 min in TBST buffer and the recombinant proteins were visualized using ECL Western blotting detection reagents and analysis system (GE Healthcare, Freiburg, Germany) according to manufacturer's instructions with high performance chemiluminescence film (GE Healthcare, Freiburg, Germany) and Optimax Typ TR developer (MS Labware, Wiesloch bei Heidelberg, Germany).

2.17 Protein Activity Assays

2.17.1 DGAT and MGAT activity assay

DGAT and MGAT activity assays were performed on the basis of (Czabany et al., 2008). Cell pellets of 50 ml expression cultures were resuspended in 1 ml DGAT assay buffer (150 mM Tris-HCl, pH 7.0; 15 mM KCl; 15 mM $MgCl_2$) and transferred to 2 ml-tubes. Samples were centrifuged for 1 min at 4 °C and maximum speed in a microcentrifuge. Supernatants were discarded and pellets were resuspended in 400 µl DGAT assay buffer. An equal amount of glass beads was added and samples were vortexed eight times for 30 s, each time followed by 30 s on ice. Then, they were centrifuged for 5 min at 4 °C and 1 000 *x g*, supernatants were transferred to fresh tubes and the lysates were assayed for protein concentration using Bradford solution as described in section 2.16.1.

200 µg of yeast cell homogenate were used per assay in a final volume of 100 µl DGAT assay buffer also containing 6 nmol [1-^{14}C]-acyl-CoA (80 000 dpm) and 10 µl DAG/MAG-CHAPS solution (0.5 mM diacylglycerol or monoacylglycerol and 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in DGAT assay buffer; The DAG/MAG-CHAPS solution was prepared beforehand by drying the diacylglycerol or monoacylglycerol moiety under streaming nitrogen, then adding CHAPS and DGAT assay buffer und treating this mixture for 15 min with ultrasound. The solution could be stored at -20 °C, prior to usage it was shaken strongly and again treated for 10 min with ultrasound). Samples were incubated at 30 °C. At time points indicated for each experiment, reactions were stopped by addition of 100 µl 0.9 % (w/v) NaCl solution and 300 µl chloroform. Lipids were extracted by shaking for 3 min and samples were centrifuged for 5 min at 11 000 *x g* to separate phases. The organic phase was transferred to a fresh tube and samples were reextracted with 300 µl chloroform. Organic phases were combined, dried under streaming nitrogen and resuspended in 20 µl chloroform/methanol (1:1, v/v).

2.17.2 LPLAT activity assays

LPLAT activity assays were performed on the basis of (Stahl et al., 2008; Stalberg et al., 2009). Cell pellets of 50 ml expression cultures were washed with 0.15 M NaCl solution, then resuspended in 1 ml ice cold LPLAT sample buffer (20 mM Tris-HCl,

pH 7.6; 1 mM EDTA) and transferred to 2 ml-tubes. Samples were centrifuged for 1 min at 4 °C and maximum speed in a microcentrifuge. Supernatants were discarded and pellets were resuspended in 400 µl LPLAT sample buffer. An equal amount of glass beads was added and samples were vortexed eight times for 30 s, each time followed by 30 s on ice. Then they were centrifuged for 5 min at 4 °C and 1 000 *x g*, supernatants were transferred to fresh tubes and lysates were assayed for protein concentration using Bradford solution as described in section 2.16.1.

For testing the forward reaction, 100 µg of yeast cell homogenate were used per assay in a final volume of 100 µl LPLAT assay buffer (25 mM Tris-HCl, pH 7.6; 0.2 M KCl) also containing 0.2 mM lyso-phospholipid (0.2 M stock solutions were dissolved in ethanol/ddH_2O (1:1, v/v) and heated to 60 °C prior to usage) and 0.1 mM [1-^{14}C]-acyl-CoA (80 000 dpm). In some reactions, total lipid extracts from 100 ml-cultures of *O. tauri* or *O. lucimarinus* were utilized as putative acyl acceptor instead of lyso-phospholipids. Samples were incubated at 23 °C or 30 °C for 5-60 min, reactions were stopped by addition of 400 µl methanol/chloroform/acetic acid (50:50:1, v/v/v) and 40 µl ddH_2O. Lipids were extracted by shaking for 3 min and samples were centrifuged for 5 min at 11 000 *x g* to separate phases. The organic phase was transferred to fresh tubes and samples were reextracted with 200 µl chloroform. Organic phases were combined, dried under streaming nitrogen and resuspended in 20 µl chloroform.

For assaying the LPLAT reverse reaction, 0.1 mM di-18:2(n-6)-PC containing 80 000 dpm di-[1-^{14}C]-18:1(n-9)-PC and 80 000 dpm *sn*-1-16:0-*sn*-2-[1-^{14}C]-18:2(n-6)-PC were mixed in 100 µl LPLAT assay buffer with 2 mM CoA, 0.2 mM 18:1(n-9)-CoA, 10 mg/ml BSA and 50 µg cell homogenate. Samples were incubated at 30 °C for 1 hour and reactions were stopped with 100 µl ethanol/acetic acid (1:1, v/v).

For testing thioesterase activity, 100 µg of yeast cell homogenate were used per assay in a final volume of 100 µl LPLAT assay buffer containing 0.1 mM [1-^{14}C]-acyl-CoA (80 000 dpm) and 10 mg/ml BSA. Samples were incubated at 30 °C for 20 min, reactions were stopped by addition of 400 µl methanol/chloroform/acetic acid (50:50:1, v/v/v) and 40 µl ddH_2O. Lipids were extracted by shaking for 3 min and samples were centrifuged for 5 min at 11 000 *x g* to separate phases. The organic phase was transferred to fresh tubes and samples were reextracted with 200 µl chloroform. Organic phases were combined, dried under streaming nitrogen and resuspended in 20 µl chloroform.

2.18 Lipid analysis

2.18.1 Lipid extraction methods

2.18.1.1 Lipid extraction from algae pellets

200 ml-algae cultures were harvested by centrifugation at 3 220 *x g* for 15 min. Supernatants were discarded and 10 µg tri-pentadecanoin were added to the algae pellets as internal standard. Lipids were extracted from pellets by vigorous vortexing in 5 ml chloroform/methanol (1:2, v/v) containing 3 % (v/v) acetic acid with 2 ml glass beads and incubating the samples overnight at 4 °C (method according to (Folch et al., 1957)). The resulting lipid extracts were filtered through cotton wool. The algae pellets were reextracted with 5 ml chloroform/methanol (2:1, v/v) containing 3 % (v/v) acetic acid and again incubated overnight at 4 °C. The resulting second lipid extracts were also filtered through cotton wool and combined with the first lipid extracts. Remaining lipids within the algae pellets were extracted by incubation for 20 min at 23 °C in 2 ml hexane. Again, the resulting supernatants were filtered through cotton wool and combined with first and second lipid extracts. After drying samples under streaming nitrogen, samples were subjected to fatty acid methyl ester (FAME) derivatization.

2.18.1.2 Lipid extraction from yeast pellets

For fast lipid extraction from yeast cultures, 10 µg tri-heptadecanoin, di-heptadecanoyl-PC, di-heptadecanoyl-PE and di-heptadecanoyl-PS, respectively, were added to cell pellets from 100 ml-expression cultures. Lipids were extracted from pellets by consecutive vigorous vortexing in 3 ml methanol and 6 ml chloroform/methanol (1:1, v/v) with 2 ml glass beads for 20 min. Samples were centrifuged at 1 500 *x g* for 5 min, lipid extracts were filtered through cotton wool. Pellets were vortexed with 3 ml hexane/diethyl ether/formic acid (65:35:1, v/v/v) and samples were incubated for 20 min at 23 °C. Resulting lipid extracts were combined with the previous ones. After drying samples under streaming nitrogen, remaining lipids were dissolved in 50 µl chloroform.

In an alternative procedure that was used for lipid extraction from yeast, cell pellets of 20 ml-expression cultures were extracted by vigorous vortexing in 5 ml chloroform/methanol (1:2, v/v) with 2 ml glass beads for 4 hours at 4 °C. Samples were centrifuged for 5 min at 1 500 *x g* and 4 °C. Supernatants were transferred to fresh tubes. Cell pellets were reextracted with chloroform/methanol (2:1, v/v) overnight at 4 °C while shaking. Samples were centrifuged again as described before and supernatants were combined with the first lipid extracts. After drying samples under streaming nitrogen, remaining lipids were dissolved in 50 µl chloroform.

Another method used was the one developed by (Ejsing et al., 2009). Cell pellets from 10 ml-expression cultures were resuspended in 1 ml 150 mM NH_4HCO_3. 10 µg tri-

decapentanoin and di-heptadecanoyl-PC were added and samples were vigorously vortexed. 5 ml chloroform/methanol (17:1, v/v) were added and samples were shaken for 2 hours at 4 °C. Phases were separated by centrifugation at 4 °C and 1 500 *x g* for 5 min. Organic phases were transferred to fresh glass tubes and samples were reextracted with 5 ml chloroform/methanol (17:1, v/v) for another hour. Organic phases were combined and dried under streaming nitrogen.

2.18.1.3 *A. thaliana* seeds

20 mg *A. thaliana* seeds were homogenized in methanol/chloroform/acetic acid (50:50:1, v/v/v). 20 µg tri-decapentanoin and di-heptadecanoyl-PC, respectively, were added as internal standards. Samples were incubated for 1 hour, then they were centrifuged for 5 min at 1 500 *x g* and supernatants were transferred to fresh tubes. Pellets were reextracted with 1 ml hexane and incubated for 20 min at 23 °C. Samples were centrifuged as described before, both lipid extracts were combined and evaporated under streaming nitrogen. Remaining lipids were resuspended in 50 µl chloroform.

2.18.2 Thin layer chromatography (TLC)

TLC was performed in 20 x 20 x 10 cm vertical glass chambers with 100 ml developing solvent and silica gel thin layer plates with the fineness 60 (Kieselgel 60, 20 x 20 cm; Merck, Darmstadt, Germany).

2.18.2.1 TLC of radioactive lipid extracts

5 µl of total lipid extracts from DGAT and MGAT activity assays were applied onto a thin layer plate and separated by a two step TLC using standard lipids for identification of different lipid classes by co-migration. First, phospholipids were separated using acetic acid methyl ester/isopropanol/chloroform/methanol/0.25 % (w/v) KCl (25:25:28:10:7, v/v/v/v/v). After the solvent had reached the center, the plate was taken out of the tank and dried. Then, the plate was put into a second chamber filled with hexane/diethyl ether/ acetic acid (80:20:1, v/v/v) as developing solvent in order to separate neutral lipids.

5 µl of total lipid extracts from LPLAT activity assays were applied onto a thin layer plate and separated by TLC using standard lipids for identification by co-migration. As developing solvent, chloroform/methanol/acetic acid/ddH_2O (85:15:10:3.5, v/v/v/v) was used.

For testing the reverse reaction in LPLAT activity assays, 20 µl per sample were directly put on a thin layer plate, dried and developed in butanol/acetic acid/ddH_2O (5:2:3, v/v/v). Radiolabelled lipids were visualized using a phosphor storage screen and a phosphoimager (*Fuji* FLA-3000, Raytest, Straubenhardt, Germany).

2.18.2.2 TLC of non-radioactive lipid extracts

Lipid extracts were applied onto thin layer plates and separated by different solvents. Neutral lipids were separated using hexane/diethyl ether/acetic acid (80:20:1, v/v/v), phospholipids were separated using chloroform/methanol/acetic acid (65:25:8, v/v/v). Phospholipids were separated from monogalactosyldiacylglycerol using acetic acid methyl ester/isopropanol/chloroform/methanol/0.25 % (w/v) KCl (25:25:25:10:9, v/v/v/v/v). Standard lipids were applied to identify the different lipid classes by co-migration.

For analytical purposes, thin layer plates were incubated in $CuSO_4$ solution (10 g $CuSO_4$ x 5 H_2O, 92 ml H_2O, 8 ml H_3PO_4) using the Chromatogram Immersion Device III (Camag, Muttenz, Switzerland). Afterwards, plates were heated on a TLC Heating Plate (Camag, Muttenz, Switzerland) to 170 °C for visualization of lipids.

For preparative purposes, thin layer plates were sprayed with 0.2 % (w/v) 8-anilino-1-naphthalene-sulphonic acid (ANS) using the TLC Spray Cabinet/Sprayer (Camag, Muttenz, Switzerland). Under UV light (λ = 254 and 365 nm) lipids were visualized and could be marked in order to scrape the desired fractions from the plate for derivatization of fatty acids.

2.18.3 Isolation and derivatization of fatty acids

2.18.3.1 Derivatization of fatty acids from yeast cell sediments

Total fatty acids (unbound and esterified to lipids) from cell pellets of 5-10 ml yeast expression cultures were derivatized into their respective FAMEs via acidic hydrolysis (Miquel and Browse, 1992). 2 ml FAME solution (2.75 % (v/v) H_2SO_4 (95-97 %) and 2 % (v/v) dimethoxy propane in methanol) were added to the washed yeast cell pellets. Samples were vortexed and incubated for 1 hour at 80 °C. Afterwards, 200 µl 5 M NaCl solution and 2 ml hexane were added. Samples were vortexed and then centrifuged at 1 500 *x g* for 5 min. The upper phase was transferred to new glass tubes and dried under streaming nitrogen. The remaining FAMEs were then dissolved in 10-50 µl acetonitrile and transferred to gas chromatography (GC) vials for subsequent analysis.

2.18.3.2 Derivatization of fatty acids from pooled seeds

Total fatty acids (unbound and esterified to lipids) from 3 mg *A. thaliana* seeds were also derivatized into their respective FAMEs based on the procedure of acidic hydrolysis (Miquel and Browse, 1992). 20-40 µg tri-decapentanoin and 2 ml FAME-toluol solution (2.75 % (v/v) H_2SO_4 (95-97 %) and 2 % (v/v) dimethoxy propane in methanol/toluol (2:1, v/v)) were added to the seeds, samples were incubated and extracted as described above (2.18.3.1). After evaporation of the solvent, methyl esters were dissolved in 200 µl acetonitrile for GC measurement.

2.18.3.3 Derivatization of fatty acids from lipid extracts or isolated lipid fractions

Fatty acids esterified to lipids were derivatized into their respective FAMEs by transesterification (Hornung et al., 2002). 333 µl toluol/methanol (1:2, v/v) and 167 µl 0.5 M $NaOCH_3$ were added to the samples, which were incubated for 20 min at 23 °C. Then, 100 µl 5 M NaCl solution were added and methyl esters were extracted with 2 ml hexane. Samples were centrifuged at 1 500 *x g* for 5 min. The upper phase was transferred to fresh glass tubes and dried under streaming nitrogen. Remaining methyl esters were then dissolved in 10-50 µl acetonitrile for GC analysis.

2.18.3.4 Derivatization of fatty acids from single *A. thaliana* or *C. sativa* seeds

Single seeds were treated with trimethylsulfoniumhydroxide (TMSH) according to (Müller et al., 1990) in order to obtain FAMEs from unbound fatty acids as well as from fatty acids esterified to lipids. Single seeds were ground in 5-20 µl TMSH in a GC vial. In case of *C. sativa* seeds, 10 µl of the resulting supernatant was transferred to a fresh vial to avoid plugging of the GC injection needle. Transesterification occurred during incubation at 23 °C. At the same time, the TMSH evaporated and the methyl esters could be dissolved in 10-20 µl acetonitrile for GC measurement.

2.18.4 Gas chromatography (GC)

FAMEs were analysed via a gas chromatograph coupled to a flame ionisation detector (FID) (6890 series GC system; Agilent, Waldbronn, Germany) using a capillary DB-23 column (30 m x 0.25 mm, 0.25 µm coating thickness, J&W Scientific, Agilent, Waldbronn, Germany) according to (Hornung et al., 2002). Helium was used as carrier gas with a flow of 1 ml/min. The temperature gradient was 150 °C for 1 min, 150-200 °C at 4 K/min, 200-250 °C at 5 K/min and 250 °C for 6 min. As retention time standards, FAME-Mix (C4-C24; Sigma, München) as well as Menhaden oil (Sigma, München) derivatized into FAMEs were injected before samples were run. Injection volumes depended on the concentration of FAMEs in the sample. Data were processed using ChemStation Rev. A09.03 (Agilent, Waldbronn, Germany).

FID signals, which could not be identified by GC were further analysed by their mass spectra using a 6890 gas chromatograph/5973 mass selective detector system (Agilent, Waldbronn). GC conditions were the same as for GC analysis. As MS conditions, an electron energy of 70 eV, an ion source temperature of 230 °C and a temperature of 260 °C for the transfer line were used. Resulting spectra were compared with the Lipid Library of the Scottish Crop Science research Institute (http://www.lipidlibrary.co.uk/index.html) to identify unknown substances.

2.18.5 Extraction, derivatization and analysis of acyl-CoAs

For analysis of acyl-CoAs from yeast pellets, the method developed by (Larson and Graham, 2001) was used. During this procedure, isolated acyl-CoA esters are converted into etheno-derivates which are then separated by reversed-phase high per-

formance liquid chromatography (HPLC) and can be detected by a fluorescence detector. Extraction of acyl-CoAs was performed on the basis of (Rosendal and Knudsen, 1992). To cell pellets from 10 ml-yeast cultures, 0.25 nmol heptadecanoyl-CoA as internal standard, 800 µl ddH_2O, 3 ml chloroform/methanol (2:1, v/v) and 2 ml glass beads were added. Samples were vigorously vortexed at 4 °C for 20 min. 1 ml chloroform and 1 ml ddH_2O were added and samples were vortexed for 30 s. Then they were centrifuged for 10 min at 3 320 *x g* and 4 °C. Upper and lower phase were discarded, the interphase was dried under streaming nitrogen. 400 µl extraction buffer (2 ml isopropanol, 2 ml 50 mM KH_2PO_4, 50 µl acetic acid, 80 µl 50 mg/ml BSA (fatty acid free, Sigma, Munich)), 10 µl saturated $(NH_4)_2SO_4$ and 1200 µl methanol/chloroform (2:1, v/v) were added to the samples. These were vortexed on a shaker at 4 °C for 20 min. Samples were then incubated for 20 min at 23 °C and afterwards centrifuged at 3 320 *x g* for 5 min. Supernatants were transferred to 1.5 ml-tubes and dried under streaming nitrogen. Samples were either frozen at -80 °C or directly derivatized by addition of 300 µl derivatization solution (50 % (v/v) chloroacetaldehyde solution (7.9 M) and 0.5 % (w/v) SDS in ddH_2O, adjusted to pH 4 with 0.15 M citrate buffer) and incubation for 20 min at 85 °C. Samples could either be measured directly or stored at -20 °C until analysis was performed.

Etheno-derivates were separated at 300 bar by using the 1100 Series HPLC system (Agilent, Waldbronn, Germany), a LUNA column (150 x 2 mm, coated with 5 µm phenyl-hexyl silicium particles; Phenomenex, Aschaffenburg, Germany) as main column, a phenyl propyl guard (4 x 2 mm) as precolumn (Phenomenex, Aschaffenburg, Germany) and a fluorescence detector (λ(excitation) = 230 nm, λ(emission) = 420 nm). 10-20 µl of each sample were injected for every measurement. Standard acyl-CoAs were also derivatized and used for identification of peaks in the samples. The gradient for separation and elution of the different etheno-derivates is given below.

Time (min)	A (%)	B (%)	C (%)	D (%)	Flow rate (ml/min)
0	90	10	0	0	0.4
5	20	80	0	0	0.4
5.1	20	0	80	0	0.4
7	0	0	97	3	0.2
10	0	0	95	5	0.2
10.1	0	0	95	5	0.2
50	0	0	55	45	0.2
51.1	0	0	0	100	0.4
52	0	0	0	100	0.4
62	0	0	0	100	0.4
62.1	90	10	0	0	0.4
65	90	10	0	0	0.4

eluent A: H_2O/acetic acid (100:1, v/v)

eluent B: acetonitrile/H_2O/acetic acid (90:9:1, v/v/v)

eluent C: H_2O/triethylamine (100:0.25, v/v)

eluent D: acetonitrile/H_2O (90:10, v/v)

3 Results

Marine microalgae are primary producers of VLCPUFAs and therefore potential gene donors for genes encoding enzymes active in VLCPUFA biosynthesis or distribution. Microalgal acyltransferases for example might be useful to improve the substrate feed for the biosynthetic enzymatic activities or to mediate transfer of the end products into a desired lipid fraction. Desaturases or elongases, on the other hand, are directly required for the synthesis of VLCPUFAs. In previous investigations, the closely related microalgal species *O. tauri*, *O. lucimarinus* and *M. squamata* turned out to be useful gene donors for both acyltransferases and desaturases (Domergue et al., 2005; Hoffmann et al., 2008; Wagner, 2008).

3.1 Characterization of microalgal acyltransferases in yeast

The prasinophyte *O. tauri* was shown to accumulate high amounts of DHA in its TAG fraction. Because of this finding, the organism was believed to be a valuable model to analyze mechanisms of VLCPUFA distribution into its neutral lipids (Wagner et al., 2010). Therefore, nucleotide sequences encoding different acyltransferases putatively involved in this mechanism were isolated by Dr. Martin Wagner. Due to its close relationship to *O. tauri*, also *O. lucimarinus* was used as gene donor for a potential acyltransferase (Wagner, 2008). The identified sequences were already partly analyzed during the beforehand mentioned work. However, experiments were still missing for the complete characterization and were thus conducted in the presented work.

3.1.1 Characterization of the putative acyl-CoA:lysophosphatidylcholine acyltransferases OlLPCATp and OtLPCATp

OtLPCAT and *OlLPCAT* were identified by searching through the genome of *O. tauri* and *O. lucimarinus* with *ALE1*, a yeast LPCAT (Benghezal et al., 2007; Chen et al., 2007; Jain et al., 2007; Riekhof et al., 2007; Tamaki et al., 2007). Over-expression of the completely codon-optimized *OlLPCAT* in yeast led to solid protein amounts detectable in Western Blot and was able to partially complement the yeast mutant strain BY4741 *ale1Δ* deficient in intrinsic LPCAT activity (Wagner, 2008). *OtLPCAT* was isolated and partly codon-optimized by Dr. Mareike Heilmann, but was not characterized so far. Therefore, it was checked first whether over-expression of *OtLPCAT* led to detectable protein levels in yeast. For this purpose, both sequences directly fused to a V5-epitope sequence at their 3'-end in pYES2/CT were expressed under the control of *GAL1* promoter in the yeast strain BY4741 *ale1Δ*. As positive control, the ß-galactosidase sequence *LacZ* fused to a V5-epitope sequence in pYES2/CT and as negative control, empty pYES2/CT vector were transformed into the same strain. Ex-

pression cultures were grown for two days at 23 °C. Afterwards, cultures were harvested and protein lysates were analyzed by Western Blot for V5-epitope. As can be seen in Figure 4 A, the positive control showed a band at 120 kDa, which represents the expected size of the ß-galactosidase protein with carboxyl-terminal V5-epitope. In contrast, protein lysates from both *OtLPCAT*- and *OlLPCAT*-expressing cultures did not exhibit bands at the expected protein sizes of 58 kDa and 50 kDa, respectively. The observed band pattern was not different from the one deriving from the negative control culture lysates and thus seemed to result from unspecific binding of the antibody. *OlLPCAT* and *OtLPCAT* sequences were also cloned into pUG36 in order to obtain amino-terminal GFP-tagged proteins. Western Blot experiments with cell lysates expressing these constructs also led to negative results (data not shown). In addition, yeast cells expressing *GFP-OlLPCAT* or *GFP-OtLPCAT* were observed under the microscope, but neither of the fusion proteins could be detected (data not shown).

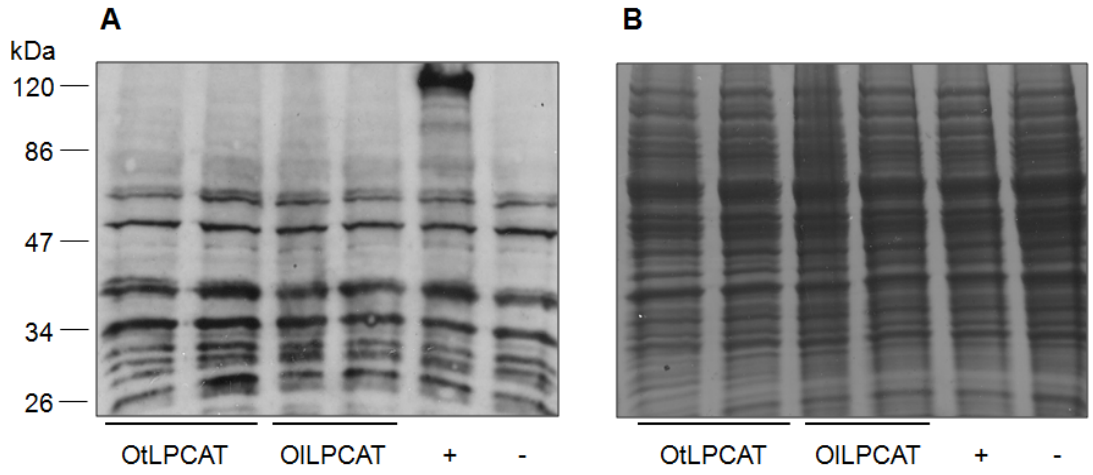

Figure 4. Protein amounts after over-expression of *OtLPCAT-V5* and *OlLPCAT-V5*

OtLPCAT-V5-pYES2/CT, OlLPCAT-V5-pYES2/CT, LacZ-V5-pYES2/CT (+; positive control) or pYES2/CT (-; negative control) were expressed for two days at 23 °C in BY4741 *ale1Δ*. Cells were lysed and total lysates were analyzed. (A) Western Blot and (B) corresponding Coomassie gel as loading control. OtLPCAT-V5p was expected to be 58 kDa, OlLPCAT-V5p was expected to be 50 kDa and LacZ-V5p 120 kDa in size. The experiment was performed once with two independent clones.

Nevertheless, in previous experiments *OlLPCAT* expression was found to partially complement the yeast mutant strain BY4741 *ale1Δ* (Wagner, 2008). Also, expression of sequences lacking the epitope sequence might result in higher protein levels in yeast compared to the V5-epitope fusions of OtLPCATp and OlLPCATp. Therefore, *in vitro* LPLAT assays were performed with cell homogenates from cultures expressing the microalgal sequences without epitope to elucidate the catalyzed reaction and substrate specificities of the potential acyltransferases. The completely codon-optimized sequence of *OlLPCAT* and the partly codon-optimized sequence of *OtLPCAT* were expressed under the control of *GAL1* promoter in pYES2/CT in the yeast strain BY4741 *ale1Δ*. As negative control, pYES2/CT was expressed in the mutant strain. As positive controls, the construct ALE1-pYES2.1/TOPO (by courtesy of Dr. U. Ståhl) in the mutant strain and pYES2/CT in BY4741 wild type cells were expressed. Expression cultures were grown either for one day at 30 °C, for two days at 23 °C or for three days at 16 °C. Afterwards, yeast cells were harvested and used for homogenate preparation. Resulting homogenates were tested with different lyso-phospholipids as acyl acceptors and various radiolabeled acyl-CoAs as acyl donors in the LPLAT activity assay. The result of an exemplary experiment is depicted in Figure 5. Whereas both positive controls exhibited considerable amounts of radiolabeled and thus newly formed PC, expression of neither *OtLPCAT* nor *OlLPCAT* could complement the PC-deficient phenotype of BY4741 *ale1Δ in vitro*. Varying expression and assay temperatures as well as different acyl donor and acceptor combinations did not result in detectable activity of the putative microalgal LPCATs. In Table 1, an overview about different combinations of acyl acceptors and acyl donors is given. Also, lipid extracts from *O. tauri* or *O. lucimarinus* were tested in some experiments as possible acyl acceptors instead of defined lyso-phospholipids, but no differences could be observed between lysates from cultures expressing *OtLPCAT* or *OlLPCAT* in comparison to those from cultures expressing empty vector (data not shown).

Also, the reverse LPLAT reaction of cell homogenates was assayed by using radiolabeled PC and CoA under excess of 18:1(n-9)-CoA. Furthermore, thioesterase activity was examined by incubation of different radiolabeled acyl-CoAs together with cell homogenates. In both assays, no catalytic activity for OtLPCATp and OlLPCATp could be shown (data not shown).

Summing up these results, both *OlLPCAT* and *OtLPCAT* did not reveal detectable protein levels upon expression in yeast when fused to the V5-epitope sequence and for both putative acyltransferases neither LPLAT nor thioesterase activity could be shown *in vitro* when the native proteins without the V5-epitope were used.

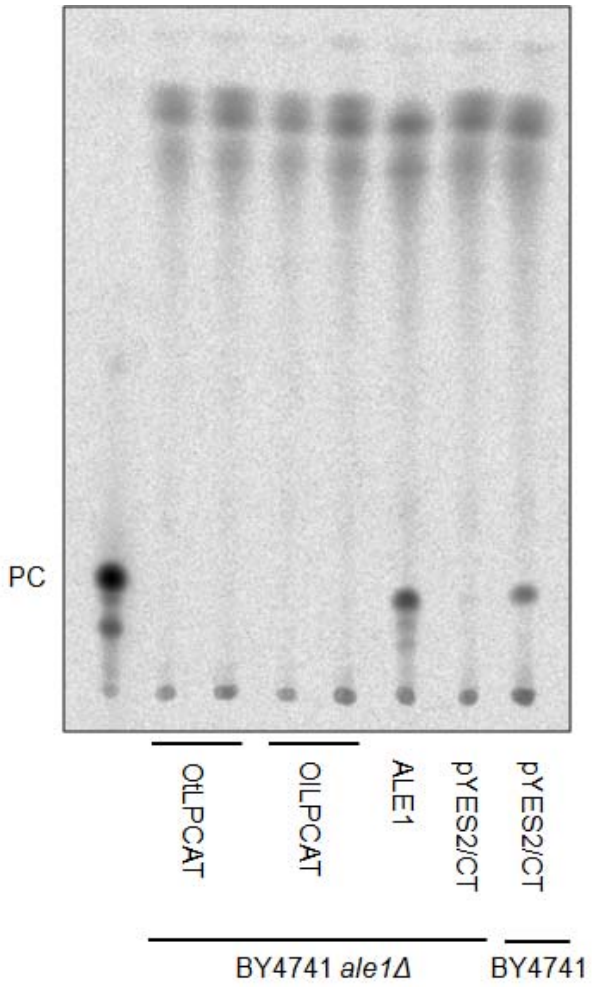

Figure 5. OtLPCATp and OlLPCATp activity *in vitro*

pYES2/CT (negative control), OtLPCAT-pYES2/CT, OlLPCAT-pYES2/CT or ALE1-pYES2.1/TOPO (positive control) were expressed for one day at 30 °C in the yeast mutant BY4741 *ale1Δ* or in the wild type strain BY4741 as indicated. Cell homogenates were prepared and incubated with [1-^{14}C]-18:3(n-3)-CoA and lyso-16:0-PC. Reactions were incubated at 30 °C and stopped after five min. PC, phosphatidylcholine. The experiment was performed once with two independent clones for OtLPCATp and OlLPCATp.

Table 1. Overview about different *in vitro* assay conditions to test for OtLPCATp and OlLPCATp activity

OtLPCAT-pYES2/CT, OlLPCAT-pYES2/CT and positive as well as negative controls were expressed for one day at 30 °C or for two days at 23 °C or for three days at 16 °C in the yeast mutant BY4741 *ale1Δ* or in the wild type strain BY4741. Cell homogenates were prepared and incubated with [1-^{14}C]-labeled CoAs and different lyso-phospholipids as indicated. Reactions were incubated at 30 °C and stopped after five min or incubated at 23 °C for one hour. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; LE, total lipid extract. Numbers of independent experiments for each putative LPCAT are indicated (multiple clones or multiple expressions of the same clone).

	18:1(n-9)-CoA	**18:3(n-3)-CoA**	**18:3(n-6)-CoA**	**20:4(n-6)-CoA**
16:0-lyso-PC	7	8		
16:0-lyso-PE	2			
16:0-lyso-PG	2			
18:0-lyso-PA	2			
O. tauri LE	2	2	2	2
O. lucimarinus LE	2	2	2	2

3.1.2 *In vitro* studies of the acyl-CoA:diacylglycerol acyltransferase OtDGAT2Bp

OtDGAT2B was identified by searching through the genome of *O. tauri* with DGAT2 nucleotide sequences from the fungus *M. ramanniana* (Lardizabal et al., 2001). Expression of the partly codon-optimized *OtDGAT2B* led to detectable protein levels in yeast and was found to complement the yeast mutant BY4741 *dga1Δ lro1Δ* lacking the only DGAT and PDAT enzymes in yeast and thus being devoid of TAG. Furthermore, *in vivo* yeast expression experiments showed that the enzyme does not differentiate neither between (n-3)- and (n-6)-substrates nor between acyl-CoAs with 18 or 20 carbon atoms (Wagner et al., 2010). The remaining question was whether *in vitro* assays would confirm the previously *in vivo* obtained results concerning substrate specificity. For this purpose, the yeast strain BY4741 *dga1Δ lro1Δ* (provided by Dr. M. Wagner) was used to additionally knockout the yeast gene for acyl-CoA thioesterase *TES1* (Jones et al., 1999; Ntamack et al., 2009) by PCR-mediated gene deletion. This was done because TES1p might have interfered with the *in vitro* assay by cleavage of the externally provided acyl-CoA substrates. After successful gene deletion, pYES2/CT-constructs containing either the partly codon-optimized *OtDGAT2B* or, as positive control, the yeast DGAT cDNA sequence *DGA1* (Oelkers et al., 2002; Sorger and Daum, 2002) under control of the *GAL1* promoter (provided by Dr. M. Wagner) were transformed into BY4741 *dga1Δ lro1Δ tes1Δ*. As negative control, the empty vector pYES2/CT was transformed into the same strain. Expression cultures were grown for one day at 30 °C. Afterwards, yeast cells were harvested and used for homogenate

preparation. Resulting homogenates were tested with different DAGs as potential acyl acceptors and various radiolabeled acyl-CoAs as potential acyl donors in a DGAT activity assay. Results of two exemplary experiments are depicted in Figure 6. Figure 6 A shows TAG formation after utilization of di-18:1(n-9)-DAG as acyl acceptor together with [1-^{14}C]-18:1(n-9)-CoA as acyl donor, whereas Figure 6 B shows results after utilization of di-18:2(n-6)-DAG together with [1-^{14}C]-18:1(n-9)-CoA. Both experiments led to a similar outcome. TAG formation was strongest after expression of endogenous *DGA1* in yeast. Even when the reaction was stopped immediately after addition of all components (0 min), TAG formation was observable for the positive control. In contrast, homogenates of *OtDGAT2B*-expressing cultures produced TAG in visible amounts no more than one hour after initiation of the experiment. In addition to [1-^{14}C]-18:1(n-9)-CoA also [1-^{14}C]-18:3(n-3)-CoA, [1-^{14}C]-18:3(n-6)-CoA as well as [1-^{14}C]-20:4(n-6)-CoA were tested as putative acyl donors. Comparable TAG formation could be observed for homogenates from *DGA1*-expressing cultures with all tested substrates. For homogenates from *OtDGAT2B*-expressing cultures, no differences in comparison to the negative control homogenates could be observed (data not shown).

In conclusion, OtDGAT2Bp *in vitro* preferred 18:1(n-9)-CoA to polyunsaturated fatty acyl-CoAs with 18 or 20 carbon atoms as acyl donor but did not differentiate between di-18:1(n-9)-DAG and di-18:2(n-6)-DAG as acyl acceptor.

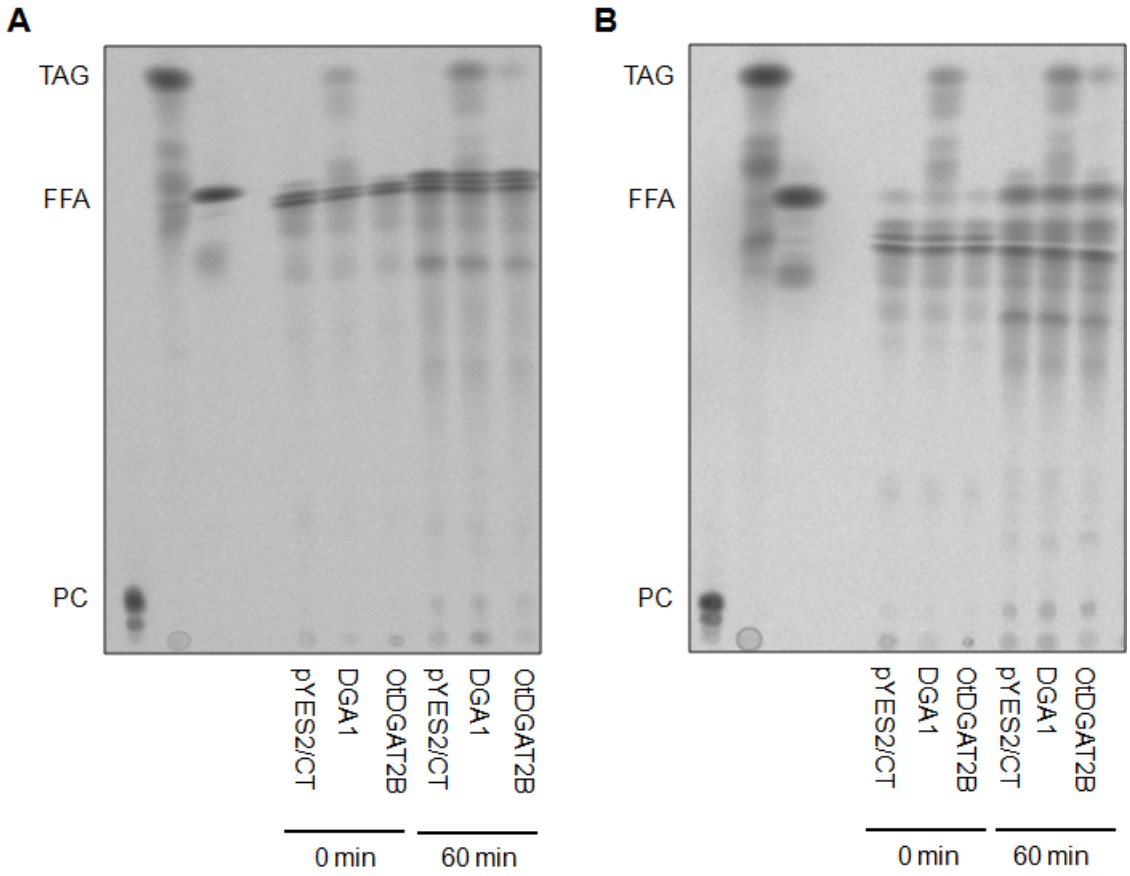

Figure 6. OtDGAT2Bp activity *in vitro*

pYES2/CT (negative control), DGA1-pYES2/CT (positive control) or OtDGAT2B-pYES2/CT were expressed for one day at 30 °C in the yeast mutant BY4741 *dga1Δ lro1Δ tes1Δ*. Cell homogenates were prepared and incubated with [1-^{14}C]-18:1(n-9)-CoA as acyl donor and (A) di-18:1(n-9)-DAG or (B) di-18:2(n-6)-DAG as acyl acceptors. Reactions were stopped at time points indicated. PC, phosphatidylcholine; TAG, triacylglycerol; FFA, free fatty acids. Presented is a representative result of (A) two or (B) one experiment(s).

3.1.3 *In vivo* and *in vitro* studies of the putative acyl-CoA:diacylglycerol acyltransferases OtDGAT2Ap and OtDGAT2Cp

OtDGAT2A and *OtDGAT2C* were identified in the same screen that also led to the identification of *OtDGAT2B*. Both sequences had to be completely codon-optimized to obtain detectable levels of protein after over-expression in yeast. In previous experiments OtDGAT2Ap and OtDGAT2Cp were not found to complement the TAG deficient yeast mutant BY4741 *dga1Δ lro1Δ* upon expression (Wagner, 2008). Because marine microalgae are adapted to life in the ocean, their enzymes might have different temperature optima compared for example to the corresponding yeast enzymes. Therefore, it the impact of expression temperature was tested. pYES2/CT-constructs containing either the completely codon-optimized nucleotide sequences of *OtDGAT2A* or *OtDGAT2C* or, as positive control, the partly codon-optimized sequence of *OtDGAT2B* under control of the *GAL1* promoter (provided by Dr. M. Wagner) were transformed into

BY4741 *dga1Δ lro1Δ*. As negative control, the empty vector pYES2/CT was transformed into the same strain. Expression cultures were grown for 29 hours at 30 °C or for 95 hours at 23 °C or 16 °C. 24 OD units of each culture were harvested, total lipids were extracted and subsequently separated by TLC. The results of the performed experiment are depicted in Figure 7. Cultures expressing the empty vector pYES2/CT did not show a spot corresponding to TAG. *OtDGAT2B*-expressing cultures produced comparably high TAG amounts under all tested expression temperatures confirming the DGAT activity of OtDGAT2Bp. In contrast, cultures expressing *OtDGAT2A* produced very low amounts of TAG only at 23 °C or even at 16 °C. Cultures expressing *OtDGAT2C* did not produce TAGs under any of the given conditions.

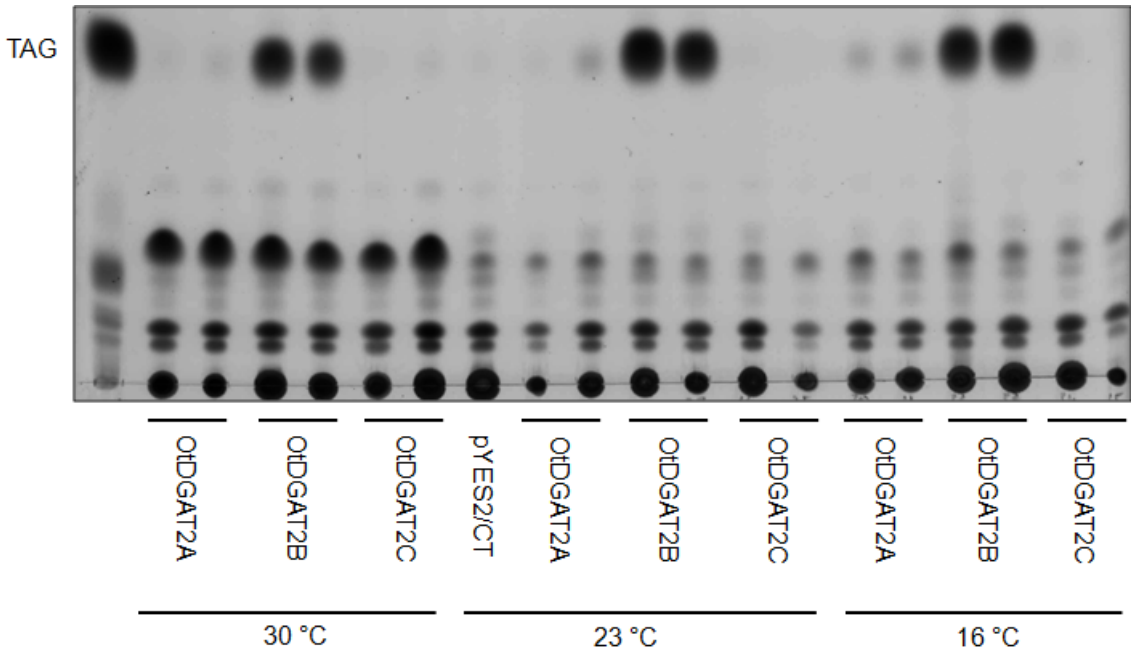

Figure 7. Complementation tests with the BY4741 *dga1Δ lro1Δ* mutant by expression of *OtDGAT2A*, *OtDGAT2B* or *OtDGAT2C*

pYES2/CT (negative control), OtDGAT2A-pYES2/CT, OtDGAT2B-pYES2/CT (positive control) or OtDGAT2C-pYES2/CT were expressed at three different temperatures as indicated. Cultures were grown either for 29 hours at 30 °C or for 95 hours at 23 °C or 16 °C, respectively. 24 OD units were harvested, total lipids were extracted and separated by TLC. TAG, triacylglycerol. The experiment was performed once with two independent clones.

Bifunctional enzymes possessing DGAT and WS activity could be identified in bacteria (Kalscheuer and Steinbüchel, 2003) and plants (King et al., 2007; Li et al., 2007). In order to test whether OtDGAT2Ap or OtDGAT2Cp possess WS activity, their codon-optimized sequences were cloned into mCherryFAR1-pESC-URA (provided by Dr. M. Heilmann) downstream of the *GAL10* promoter. The mouse fatty acid reductase (FAR1p) has been shown to reduce fatty acids bound to CoA into fatty alcohols (Cheng and Russell, 2004a). Mouse WS (provided by Dr. M. Heilmann) was used in the following experiment as positive control (Cheng and Russell, 2004b). Constructs harbouring *mCherryFAR1* alone as negative control or in combination with mouse *WS* or *Ot-*

DGAT2A or *OtDGAT2C*, respectively, were transformed into the H1246 strain (by courtesy of Prof. Dr. S. Stymne) deficient in the TAG-producing enzymes DGA1p and LRO1p (Oelkers et al., 2000) and also lacking acyl-CoA:sterol acyltransferases ARE1p and ARE2p (Yang et al., 1996; Yu et al., 1996; Zweytick et al., 2000; Valachovič et al., 2001). This strain was used because of its lack of TAG and steryl esters (Sandager et al., 2002) and thus does not interfere with the assay. Expression cultures were either grown for 46 hours at 30 °C or 23 °C or for 116 hours at 16 °C. 34 OD units of each culture were harvested, total lipids were extracted and subsequently separated by TLC. The results of the performed experiment are depicted in Figure 8. Whereas the positive control shows wax ester accumulation for all expression conditions, those cultures co-expressing *OtDGAT2A* or *OtDGAT2C* together with *FAR1* did not reveal any wax esters in their total lipids.

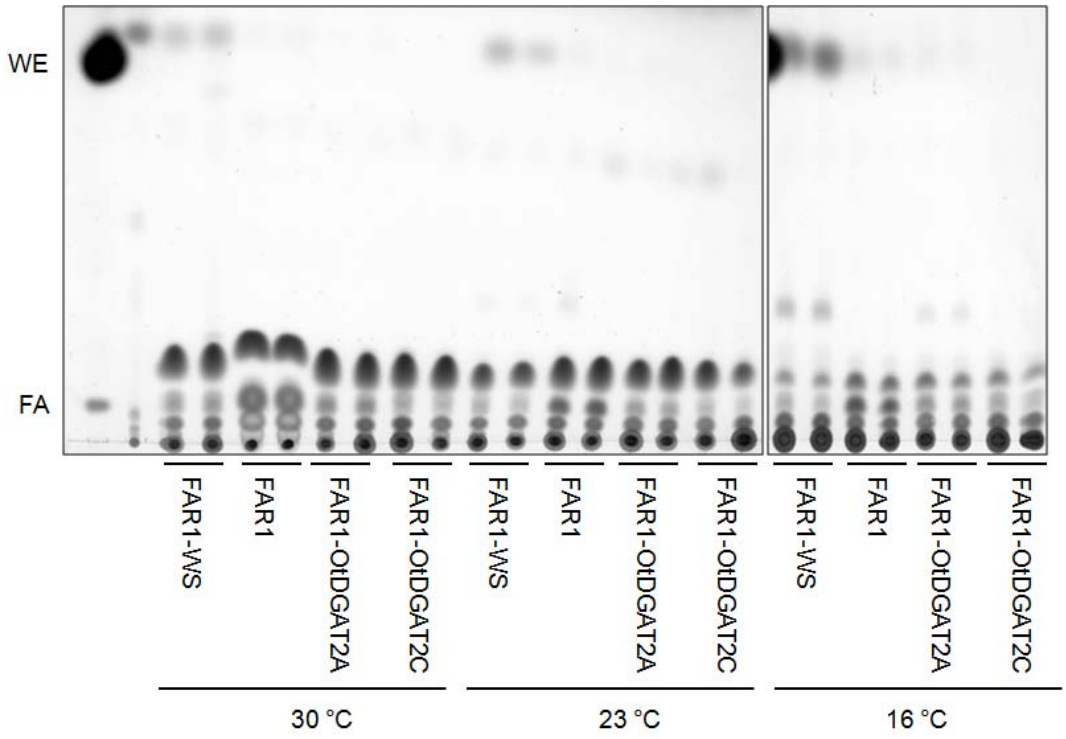

Figure 8. WS test in yeast strain H1246 by co-expression of mouse *FAR1* with *OtDGAT2A*, *OtDGAT2C* or mouse *WS*

mCherryFAR1-pESC-URA (negative control), mCherryFAR1-OtDGAT2A-pESC-URA, mCherryFAR1-OtDGAT2C-pESC-URA or mCherryFAR1-WS-pESC-URA (positive control) were expressed at three different temperatures as indicated. Cultures were grown for 46 hours at 30 °C or 23 °C or for 116 hours at 16 °C. 34 OD units of each culture were harvested, total lipids were extracted and subsequently separated by TLC. WE, wax esters; FA, fatty alcohols. The experiment was performed once with two independent clones.

In order to test whether OtDGAT2Ap and OtDGAT2Cp revealed enzymatic activity *in vitro*, experiments similar to the assays performed for OtDGAT2Bp were conducted. pYES2/CT-constructs containing either codon-optimized *OtDGAT2A*, *OtDGAT2C* or, as positive control, the sequence of *DGA1* under control of the *GAL1* promoter were transformed into BY4741 *dga1Δ lro1Δ tes1Δ*. As negative control, the empty vector pYES2/CT was transformed into the same strain. Expression cultures were grown and homogenates were prepared as described above. The assay was performed with di-18:1(n-9)-DAG as acyl acceptor and [1-^{14}C]-18:1(n-9)-CoA or [1-^{14}C]-20:4(n-6)-CoA as acyl donor. Results of the experiment are depicted in Figure 9. For homogenates from cultures expressing *DGA1*, TAG formation independent of the acyl donor could be observed. In contrast, homogenates from cultures expressing *OtDGAT2A* and *OtDGAT2C* did not show any DGAT activity in the assay.

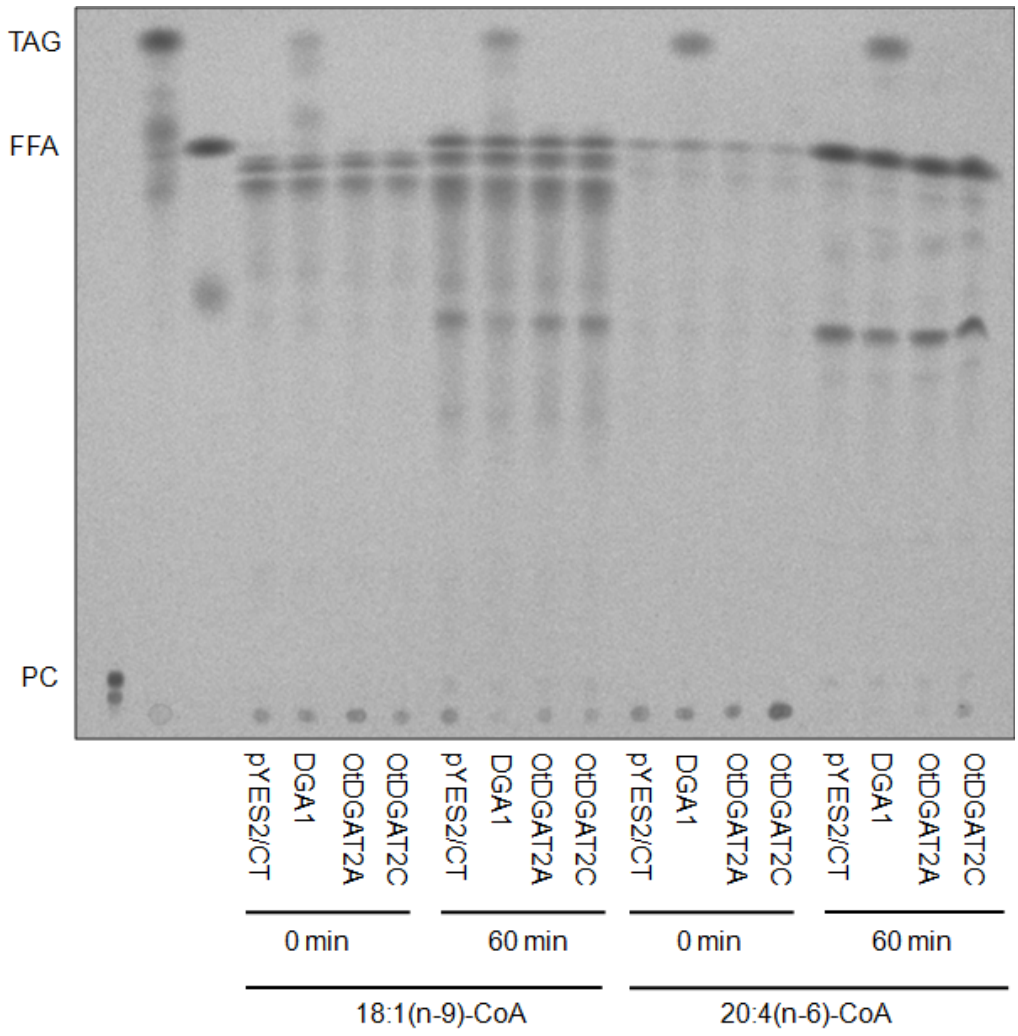

Figure 9. OtDGAT2Ap and OtDGAT2Cp activity *in vitro*

pYES2/CT (negative control), DGA1-pYES2/CT (positive control), OtDGAT2A-pYES2/CT or OtDGAT2C-pYES2/CT were expressed for one day at 30 °C in the yeast mutant BY4741 *dga1Δ lro1Δ tes1Δ*. Cell homogenates were prepared and incubated with [1-^{14}C]-18:1(n-9)-CoA or [1-^{14}C]-20:4(n-6)-CoA and di-18:1(n-9)-DAG. Reactions were stopped at time points indicated. PC,

phosphatidylcholine; TAG, triacylglycerol; FFA, free fatty acids. Presented is the result of one experiment.

Because OtDGAT2Ap and OtDGAT2Cp share sequence similarities not only with plant and animal DGATs, but also with animal MGATs (Wagner, 2008), it was tested as well whether the microalgal proteins exhibit MGAT activity *in vitro*. For this purpose, the assay was performed as described above, but under utilization of 18:1(n-9)-MAG instead of di-18:1(n-9)-DAG as acyl acceptor. As putative acyl donors, again [1-^{14}C]-18:1(n-9)-CoA and [1-^{14}C]-20:4(n-6)-CoA were used. For both enzymes, however, MGAT activity was not detectable (data not shown).

In summary, *OtDGAT2A* expression was able to complement a TAG-deficient yeast mutant under low expression temperatures *in vivo*. For OtDGAT2Cp, TAG complementation was not observable for any of the tested temperatures. Co-expression studies with both nucleotide sequences did not reveal WS activity and assays with radiolabeled substrates did not show DGAT or MGAT activity of the microalgal enzymes *in vitro*.

3.1.4 Co-expression studies of different microalgal acyltransferase sequences

The microalgal enzymes were further investigated by co-expressing different combinations in yeast to find out whether the acyltransferases possibly interact with each other or even are only active within a complex of different acyltransferases. This was tested by analyzing total amounts and fatty acid composition of different lipid classes and by comparison of the obtained data with those from cultures expressing one acyltransferase individually. Co-localization could be shown for example for the desaturase SCD1p and the acyltransferase DGAT2p from mouse (Man et al., 2006a). LPCAT in turn might potentially interact with both DGAT and PDAT enzymes because it mediates the transfer between acyl-CoA pool on the one hand and PC pool on the other hand. Therefore, combinations of LPCATp with PDATp or DGATp were chosen for the following experiments. For co-expression with *OtLPCAT* and *OlLPCAT*, the sequence encoding the most active DGAT protein from *O. tauri*, *OtDGAT2B*, was chosen. In addition, *OtPDAT* from *O. tauri* was utilized. The nucleotide sequence was isolated by Dr. M. Wagner after searching through the genome of *O. tauri* with known PDAT sequences from yeast and *Arabidopsis*. Expression of a partly codon-optimized version of *OtPDAT* resulted in solid protein amounts in yeast. The enzyme was shown to complement the BY4741 *lro1Δ dga1Δ* mutant only after addition of (n-3)- and (n-6)-polyunsaturated fatty acids with 18 or 20 carbon atoms (Wagner, 2008).

The yeast mutant BY4741 *lro1Δ dga1Δ ale1Δ* was created to avoid interference of the endogenous yeast acyltransferases with those of microalgal origin. The completely codon-optimized *OlLPCAT* and the partly codon-optimized *OtLPCAT* were cloned into pESC-LEU downstream of the *GAL1* promoter. Subsequently, pESC-LEU, OlLPCAT-pESC-LEU or OtLPCAT-pESC-LEU were co-transformed with OtDGAT2B-pYES2/CT, OtPDAT-pYES2/CT or pYES2/CT into the newly created yeast mutant strain. As wild type control, BY4741 was co-transformed with pESC-LEU and pYES2/CT. Expression

cultures were grown for two days at 23 °C and subsequently harvested. Half of each sample was used for acyl-CoA extraction and measurement whereas the other half was utilized for total lipid extraction and separation of TAG from PC. Internal standards were used for quantification. Results of the performed experiment are depicted in Figure 10, Figure 11 and Figure 12. Acyl-CoA amounts were higher in the mutant compared to the wild type control, but this effect was not statistically significant (Figure 10 A). In all mutant expression cultures, 16:1(n-9)-fractions were elevated two to three times and percentages of 18:0 and 18:1(n-9) were lowered in comparison to wild type (Figure 10 B). TAGs were detectable only in those mutant cultures expressing *OtDGAT2B* (Figure 11 A), but the fatty acid composition of the TAG fraction from complemented mutant yeast cultures did not differ from wild type (Figure 11 B). In case of *OtPDAT* expression, TAG levels were not significantly higher than in the negative control, the yeast mutant strain co-expressing pESC-LEU and pYES2/CT (Figure 11 A). Low TAG levels in these cases did not lead to reliable data concerning fatty acid composition because of detection limitations in GC measurements. PC amounts were higher in the mutant yeast strain but this effect again was not statistically significant, as can be seen in Figure 12 A. Fatty acid composition of PC was nearly the same in all tested expression cultures (Figure 12 B).

Generally it should be noted, that strong differences in total amounts as well as in fatty acid composition could be observed already between cultures co-expressing the same combination of acyltransferase nucleotide sequences. Therefore, it might be problematic to come to a conclusion based on the presented data.

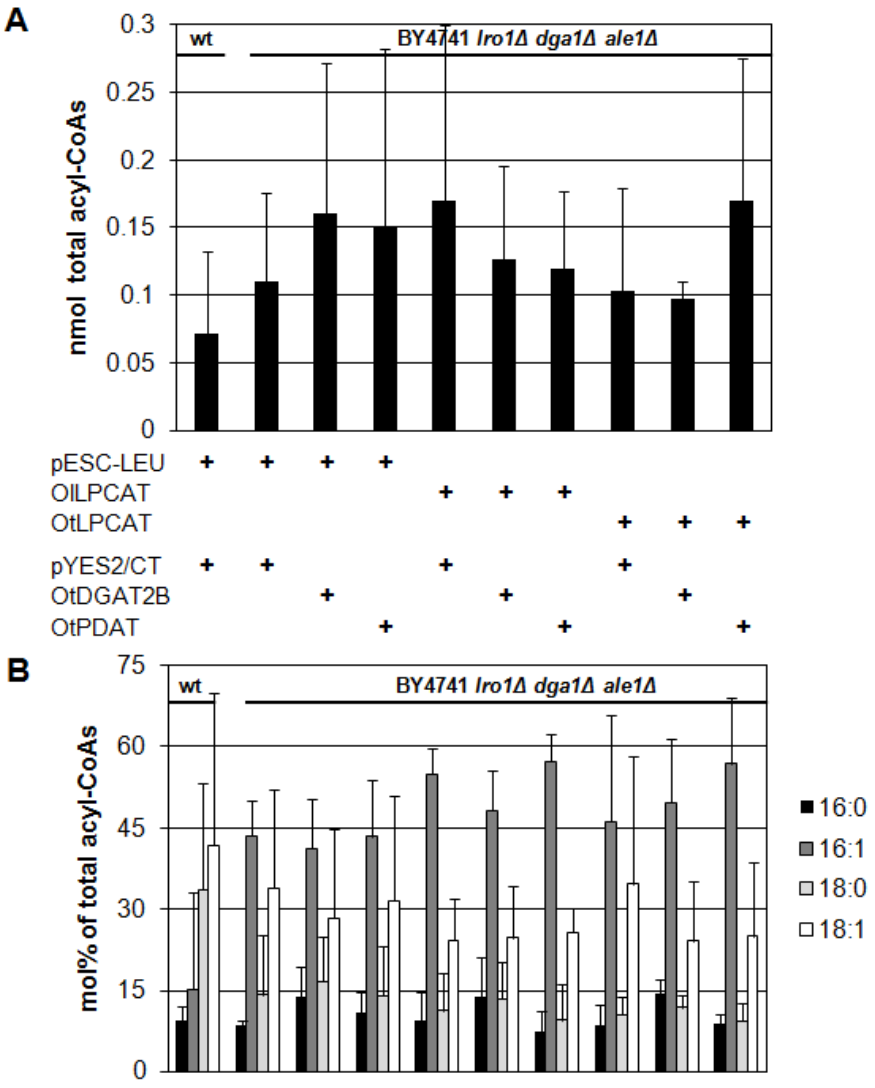

Figure 10. Acyl-CoAs after co-expression of microalgal acyltransferases in BY4741 *lro1Δ dga1Δ ale1Δ*

pESC-LEU, OlLPCAT-pESC-LEU or OtLPCAT-pESC-LEU were co-expressed with pYES2/CT, OtDGAT2B-pYES2/CT or OtPDAT-pYES2/CT as indicated between the diagrams for two days at 23 °C in the yeast mutant strain or in BY4741 (wt). Yeast cells were harvested, acyl-CoAs were extracted and measured. (A) total amounts and (B) mol% of different acyl chains. Acyl-CoA, acyl-Coenzyme A. Bars represent the mean + standard deviation (SD) of three to four independent clones.

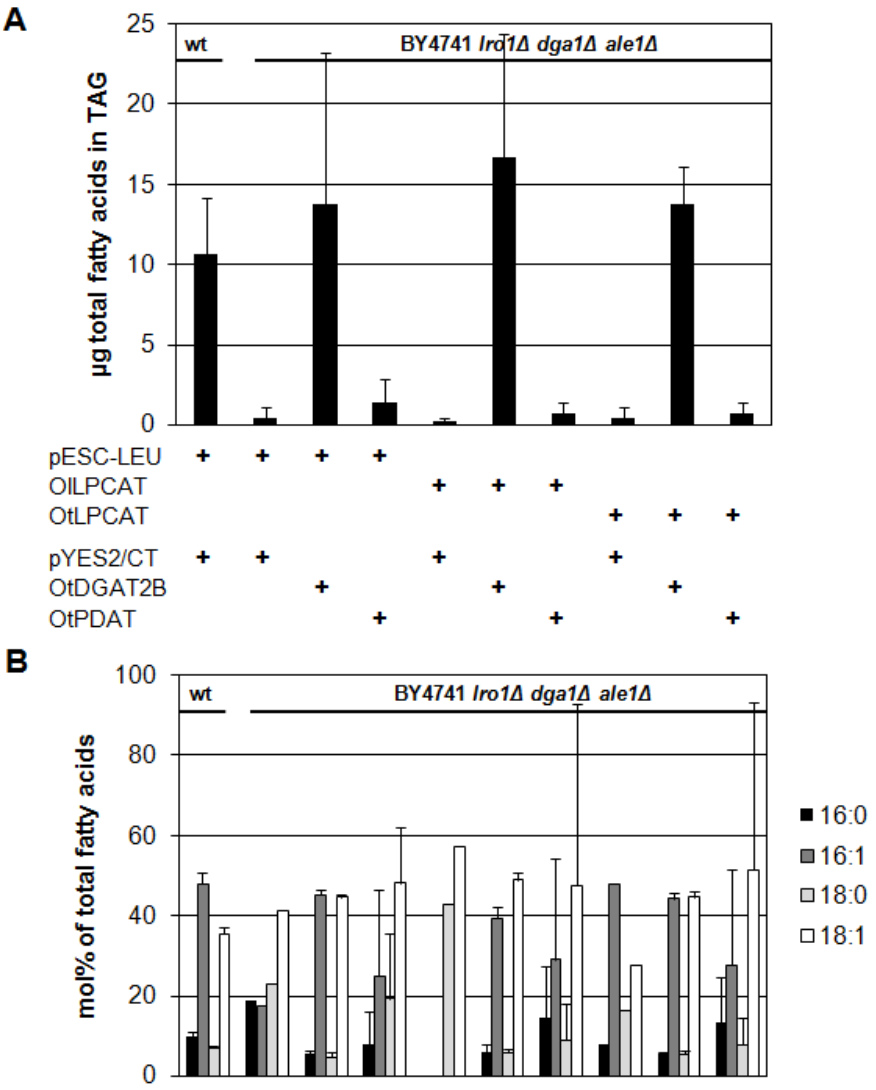

Figure 11. TAG after co-expression of microalgal acyltransferases in BY4741 *lro1Δ dga1Δ ale1Δ*

pESC-LEU, OlLPCAT-pESC-LEU or OtLPCAT-pESC-LEU were co-expressed with pYES2/CT, OtDGAT2B-pYES2/CT or OtPDAT-pYES2/CT as indicated between the diagrams for two days at 23 °C in the yeast mutant strain or in BY4741 (wt). Yeast cells were harvested, total lipids were extracted and TAGs were analyzed. (A) total amounts and (B) mol% of different acyl chains. TAG, triacylglycerol. Bars represent the mean + SD of three to four independent clones.

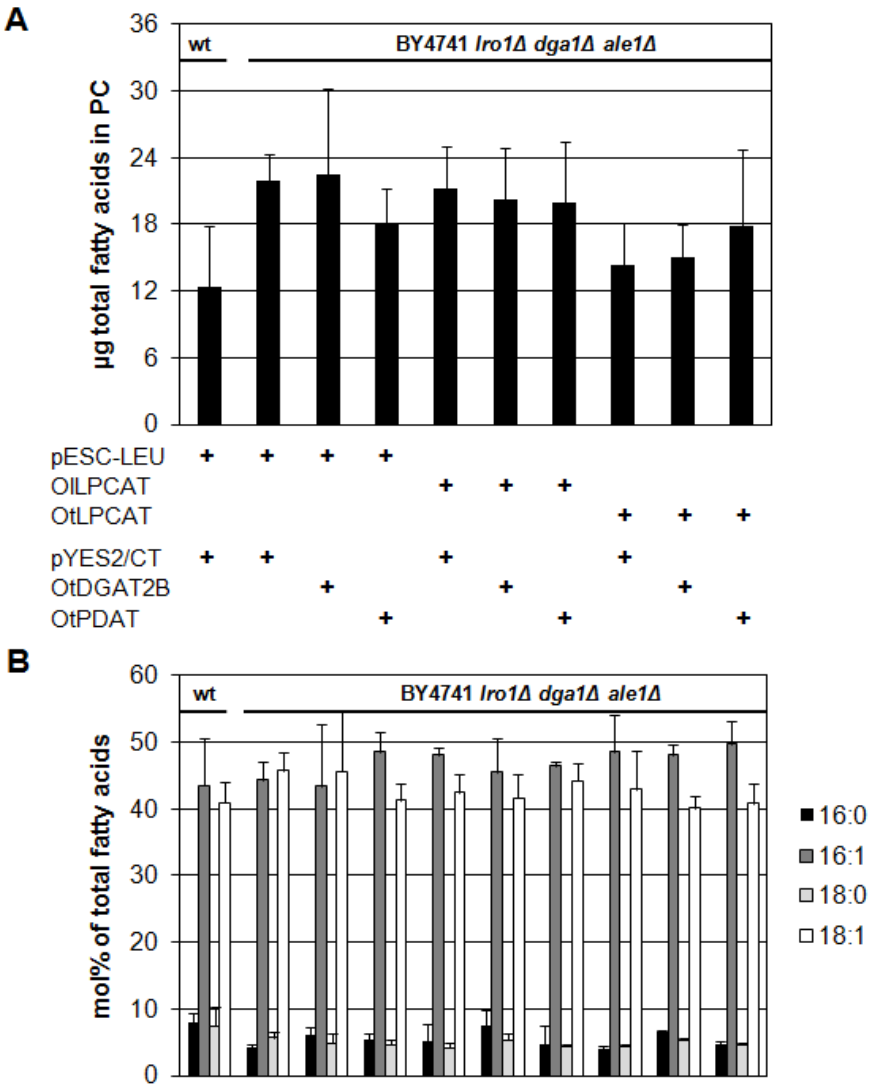

Figure 12. PC after co-expression of microalgal acyltransferases in BY4741 *lro1Δ dga1Δ ale1Δ*

pESC-LEU, OlLPCAT-pESC-LEU or OtLPCAT-pESC-LEU were co-expressed with pYES2/CT, OtDGAT2B-pYES2/CT or OtPDAT-pYES2/CT as indicated between the diagrams for two days at 23 °C in the yeast mutant strain or in BY4741 (wt). Yeast cells were harvested, total lipids were extracted and PC was analyzed. (A) total amounts and (B) mol% of different acyl chains. PC, phosphatidylcholine. Bars represent the mean + SD of three to four independent clones.

3.2 Characterization of the microalgal desaturase Old4p in yeast

In previous studies, acyl-CoA-dependent front-end desaturases with Δ6- and Δ5-specificity were identified from *O. tauri* and *M. squamata* (Domergue et al., 2005; Hoffmann et al., 2008). Their corresponding cDNA sequences were tested *in planta* and led, upon co-expression with the elongase sequence *PSE1* (Zank et al., 2002), to EPA production in *Arabidopsis* seeds. In order to establish exclusively CoA-dependent DHA synthesis, the additional implementation of an acyl-CoA-dependent desaturase with Δ4-specificity as well as an elongase with Δ5-specificity would be required. Because Δ5-elongases always act in an acyl-CoA-dependent way (Domergue et al., 2003) and have already been isolated from a few microalgae (Meyer et al., 2004; Pereira et al., 2004b), the focus was put on the identification of a Δ4-desaturase. *O. lucimarinus* in this respect appeared as a promising gene donor because it is closely related to *O. tauri* and *M. squamata* and therefore might also possess acyl-CoA-dependent desaturases.

3.2.1 Investigation of the potential gene donor *O. lucimarinus*

In order to test whether *O. lucimarinus* would be a useful candidate for isolation of a Δ4-desaturase encoding cDNA, the fatty acid composition of total lipids from *O. lucimarinus* cultures was examined first. Fatty acid profile analysis revealed sound amounts of 16:4(n-3) and DHA (Figure 13). 16:4(n-3) accounted for 14 % of total fatty acids, DHA amounts were with 3 % less pronounced. Another remarkable observation was the predominant accumulation of VLCPUFAs from the (n-3)-series that was also observable for *M. squamata* and *O. tauri* (Hoffmann, 2008). The fatty acid profile of *O. lucimarinus* thus showed several precursors of DHA which indicates that VLCPUFA synthesis in this organism occurs via sequential desaturation and elongation, but not via a polyketide synthase-like system which is characterized by releasing only trace amounts of intermediates (Truksa et al., 2009). Taken together, these results suggested *O. lucimarinus* as suitable gene donor for a Δ4-desaturase sequence.

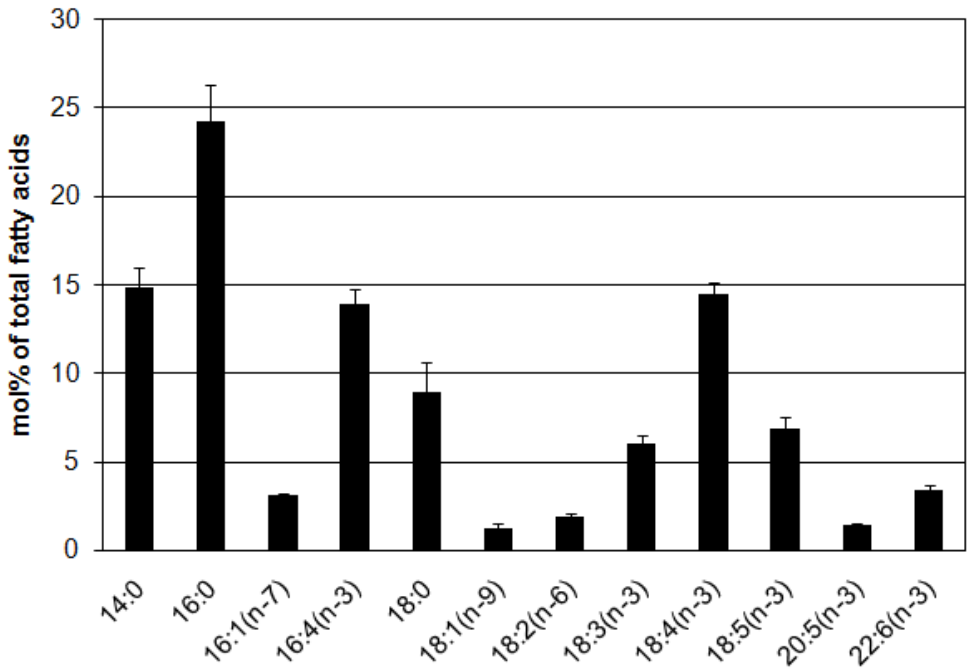

Figure 13. Fatty acid composition of total lipid extracts from *O. lucimarinus*

Cells were harvested from liquid cultures and total lipids were extracted. Fatty acids were derivatized into their respective methyl esters. These were analyzed and identified via GC-FID and GC-MS. Bars represent the mean + SD of five independent samples.

3.2.2 Identification of a putative front-end desaturase with Δ4-specificity

The microalgal genome, which had been sequenced recently by (Palenik et al., 2007), was scanned via BLAST search with already known front-end desaturases as queries for new putative desaturase sequences (Altschul et al., 1997). The identified nucleotide sequence (GenBank accession number XM_001415706) encoded a polypeptide of 459 amino acids (GenBank accession number XP_001415743) which was then further analyzed by similarity searches in the NCBI database using the BLAST program. Hits with experimentally proven substrate specificity were chosen for global pairwise alignment with the putative protein sequence Old4p. Old4p showed highest identity to two Δ4-desaturases from *Thraustochytrium sp.* (Qiu et al., 2001; Liu et al., 2007) with identity values of 28 % and 28.2 %, respectively, in pairwise alignment (Table 2). Also, Δ4-desaturases from *E. gracilis* (Meyer et al., 2003) and T. *pseudonana* (Tonon et al., 2005b) shared high identity values of 25.8 % and 25.3 %, respectively, with Old4p. As can be seen from Table 2, also several desaturases with Δ5- or Δ8-specificity were found to be similar to Old4p.

Table 2. Results from the pairwise alignment of Old4p with related proteins

Sequences are arranged according to their appearance in the BLAST search.

Related protein	Accession number	Identity (%)	Similarity (%)
Thraustochytrium sp. Δ4-desaturase (Tsd4_1p)	AAM09688	28.2	42.9
Thraustochytrium sp. Δ4-desaturase (Tsd4_2p)	AAZ43257	28	42.7
Euglena gracilis Δ4-desaturase (Egd4p)	AAQ19605	25.8	38.5
Thalassiosira pseudonana Δ4-desaturase (Tpd4p)	AAX14506	25.3	39.9
Oblongichytrium sp. Δ5-desaturase (Osd5p)	BAG71007	26.7	40.3
Marchantia polymorpha Δ5-desaturase (Mpd5p)	AAT85663	25.7	42.9
Mortierella alpina Δ5-desaturase (Mad5_1p)	BAD95486	26.8	40.2
Mortierella alpina Δ5-desaturase (Mad5_2p)	AAC72755	26.6	40.1
Thalassiosira pseudonana Δ8-desaturase (Tpd8p)	AX14502	25.6	42.3
Mantoniella squamata Δ5-desaturase (Msd5p)	CAQ30478	26.8	41.6
Phaeodactylum tricornutum Δ5-desaturase (Ptd5p)	AAL92562	27.7	41.8
Pavlova lutheri Δ4-desaturase (Pld4p)	AAQ98793	25.7	39.9

A phylogenetic tree of the chosen sequences was generated using the program Phylip (Figure 14). A multiple alignment was created with the Δ4-desaturases found via BLAST search using the online softwares MultAlin and Boxshade 3.21. In Figure 15, the multiple alignment of Old4p with its related sequences is depicted in extracts. Old4p has three histidine boxes that are necessary for coordination of two iron atoms as redox unit for the catalytical center of the desaturase (Shanklin et al., 1994; Shanklin and Cahoon, 1998). The histidine boxes (marked with continuous lines) have the sequences HX_3H, HX_3H_2 and QX_2H_2. Hence, the last box shows an H to Q substitution typical for front-end desaturases (Sperling and Heinz, 2001). In addition to that, it contains an aminoterminal HPGG-motif (marked with a dashed line) which indicates a fused cytochrome b_5 domain also characteristic for microsomal desaturases (Sperling et al., 1995; Mitchell and Martin, 1997; Napier et al., 1997).

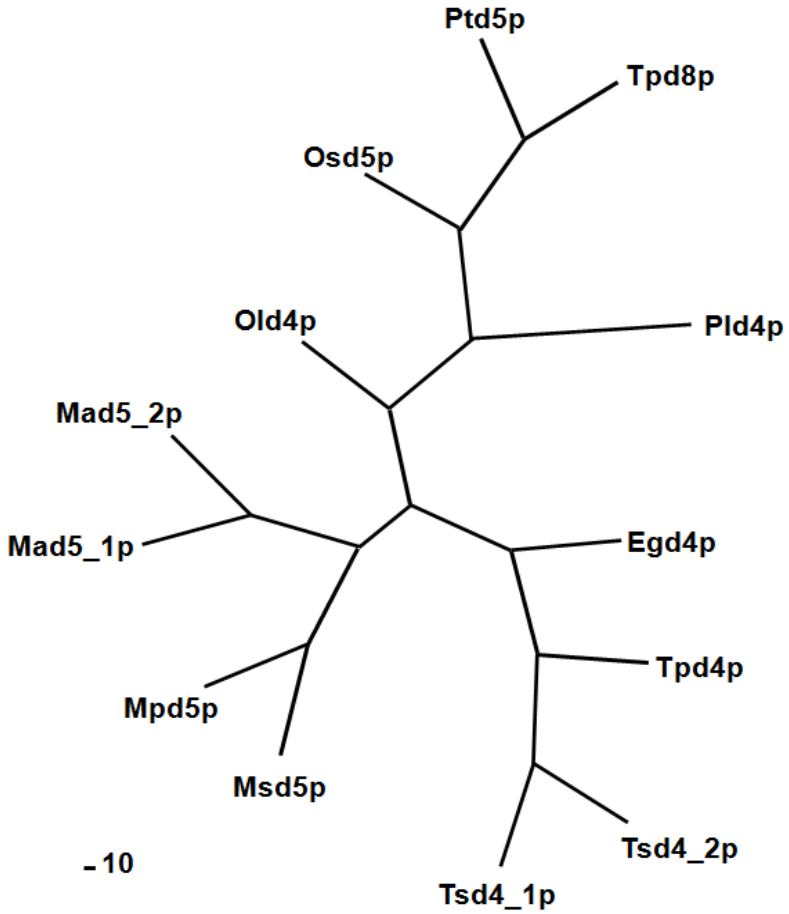

Figure 14. Phylogram of Old4p and selected functionally characterized Δ4-, Δ5- and Δ8-desaturases

The phylogram was calculated based on aligned amino acid sequences using the CLUSTALX multiple alignment and the Phylip program. Details concerning GenBank accession numbers and organisms are given in Table 2.

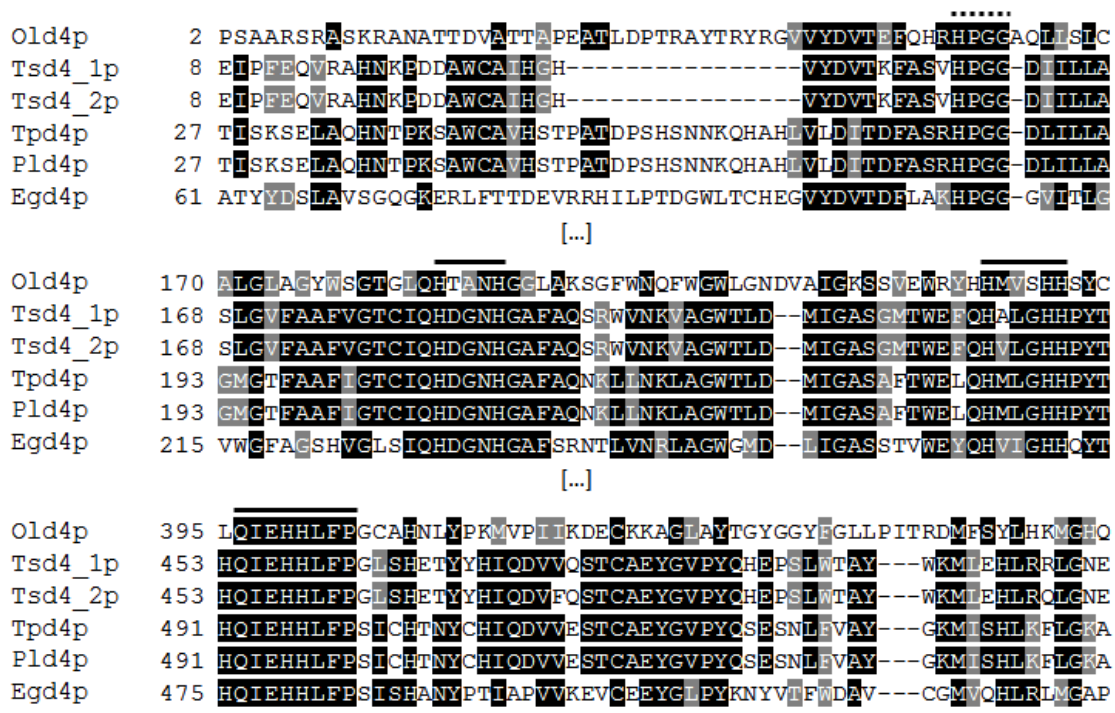

Figure 15. Partial alignment of the deduced amino acid sequence for Old4p with related Δ4-desaturases

Identical residues are shaded in black, conserved residues are shaded in grey. The three histidine boxes are marked with a continuous line. The conserved amino acid residues of the cytochrome b_5 domain are marked with a dashed line. Details concerning GenBank accession numbers and organisms are given in Table 2.

3.2.3 Catalytic activity and substrate specificities of the desaturase Old4p

In order to investigate the enzymatic activity of Old4p, the full-length nucleotide sequence of *Old4* was isolated from genomic DNA (provided by Dr. M. Heilmann), partly codon-optimized and cloned into the yeast expression vector pESC-TRP downstream of the galactose-inducible promoter GAL10. The resulting construct was transformed into the yeast strain INVSc1. As negative control, empty pESC-TRP vector was transformed into yeast. As positive control, the Δ4-desaturase nucleotide sequence *Egd4* from *E. gracilis* (Meyer et al., 2003) was isolated (provided by S. Schlenczek), cloned into pESC-TRP and transformed into INVSc1. Yeast is approved as suitable expression host for desaturase substrate specificity tests (Michaelson et al., 1998; Domergue et al., 2002; Whitney et al., 2003). Although expression in yeast might not always resemble cofactor and lipid composition of the gene donor organism (Heilmann et al., 2004), it might still allow conclusions for enzyme function. Because yeast contains only a limited number of endogenous fatty acids, namely 16:0, 16:1(n-7), 18:0, 18:1(n-9) and 18:1(n-7), substrate specificity of hypothetical desaturases was tested by addition of different free fatty acids as substrates to yeast cultures. Expression cultures were grown for three days at 23 °C. Afterwards, cultured cells were harvested, their fatty acids were derivatized into the respective FAMEs and analyzed by GC. Desaturation activity of Old4p was detected only in presence of 22:4(n-6), 22:5(n-3) and 16:3(n-3). In

Figure 16, an exemplary FAME profile of INVSc1 supplemented with 22:5(n-3) and expressing either the empty vector control, *Old4* or *Egd4* is depicted. Whereas in the control solely the supplemented substrate is observable, cultures expressing *Old4* or *Egd4* produce the desaturation product 22:6(n-3). Product identity was verified by GC-FID via coelution with standard fatty acids as well as by GC-MS.

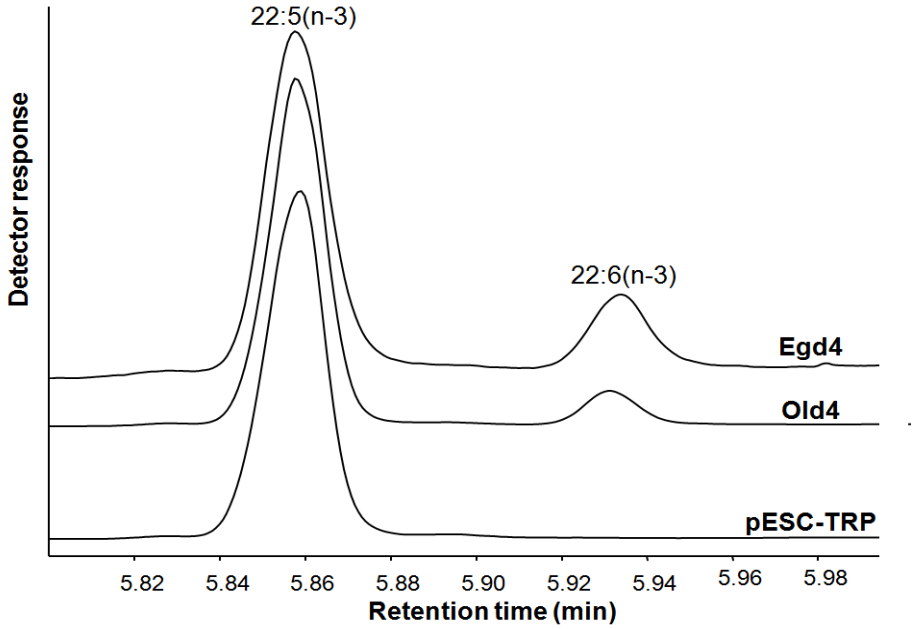

Figure 16. Gas chromatograms after expression of *Old4* and *Egd4* in yeast

FAMEs were prepared from INVSc1 cultures supplemented with 200 µM 22:5(n-3) after expression of pESC-TRP (negative control), Old4-pESC-TRP (Old4) or Egd4-pESC-TRP (Egd4; positive control) for three days at 23 °C. Product identity was verified by GC-FID and by GC-MS. Representative results of three independent experiments are shown.

In Figure 17, desaturation efficiencies of cultures expressing *Old4* or *Egd4* are compared. In these experiments, the preferred substrate of Old4p with a desaturation activity of about 10 % was 22:5(n-3) followed by 22:4(n-6) with an efficiency of about 4 %. 16:3(n-3) was desaturated only very poorly with an efficiency of about 1 %. Egd4p showed the strongest activity of 15 % on 16:3(n-3) and desaturated 22:5(n-3) and 22:4(n-6) with efficiencies of 12 % and 4 %, respectively. It should be stated that the original version of *Old4* upon expression in yeast did not lead to any desaturation products. The reason for this might be abortion of mRNA translation in yeast due to the lack of appropriate tRNAs. However, also expression of a completely codon-optimized synthetic nucleotide sequence encoding the same polypeptide as Old4 did not further enhance desaturation activity in yeast compared to the partly codon-optimized version. This finding shows that translation efficiency did not seem to be the only limiting factor for the microalgal desaturase activity in yeast.

In a nutshell, Old4p desaturated both (n-3)- and (n-6)-substrates. The enzyme furthermore preferred VLCPUFAs to a shorter-chain substrate.

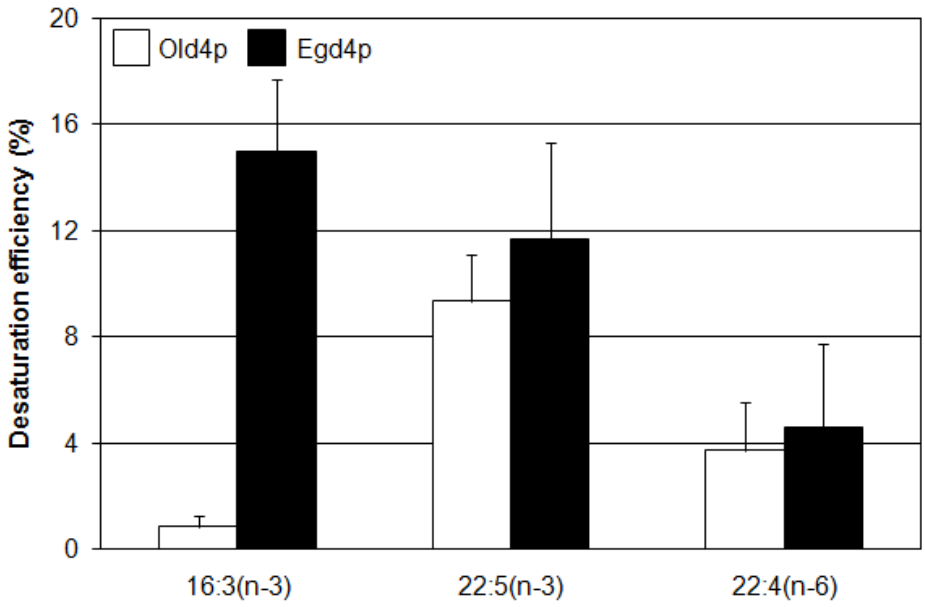

Figure 17. Conversion rates of supplied fatty acids by Old4p and Egd4p in yeast

Old4-pESC-TRP or Egd4-pESC-TRP were co-expressed with pESC-LEU for three days at 23 °C in INVSc1 cells supplemented with 200 µM of the respective fatty acid. FAMEs from yeast cells were prepared and analyzed by GC-FID and GC-MS. Desaturation efficiency was calculated as 100*product/(substrate + product). Bars represent the mean + SD of three independent experiments.

3.2.4 Distribution of Old4p and Egd4p desaturation products in different yeast lipid classes

From the distribution of desaturation product between the different lipid classes it can be deduced whether the tested desaturase acts in an acyl-CoA-dependent or in a lipid-dependent manner (Domergue et al., 2003; Hoffmann et al., 2008). Therefore, the fatty acid composition of the different lipid classes from yeast cultures expressing *Old4* or *Egd4* was analyzed (Figure 18). In both cases, more than 50 % of the desaturation product was present in the PC fraction of the cells. About 40 % of product was found in the TAG fraction, and only trace amounts of DHA could be identified in the PE or PS and PI fractions. The latter two fractions were inseparable by the utilized fractionation method. Hence, they are depicted in a combined way. These results are similar to desaturation product distributions found for the lipid-dependent desaturases Ptd5p and Ptd6p (Domergue et al., 2003; Hoffmann et al., 2008).

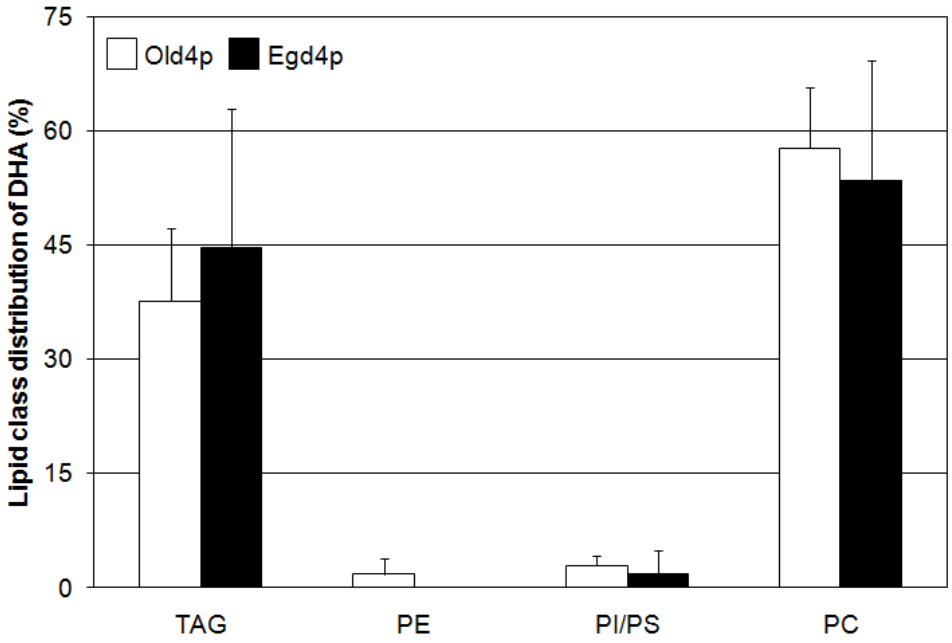

Figure 18. Lipid class distribution of DHA after action of Old4p or Egd4p in yeast

Old4-pESC-TRP or Egd4-pESC-TRP were expressed for three days at 23 °C in INVSc1 cells supplemented with 200 µM 22:5(n-3). Total lipids were extracted from yeast cultures and separated by TLC. Then they were derivatized into their respective FAMEs and analyzed by GC. TAG, triacylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine. Bars represent the mean + SD of three independent experiments.

Nevertheless, *O. lucimarinus* is a close relative of *O. tauri* and *M. squamata*, which both have been shown to contain acyl-CoA-dependent desaturases with Δ5- and Δ6-specificity (Domergue et al., 2005; Hoffmann et al., 2008). To check whether the low desaturation activity of Old4p was caused by insufficient substrate amounts in the form of acyl-CoAs, co-expression experiments were performed with a long-chain acyl-CoA synthetase from *T. pseudonana* (TpLACSp) under addition of 22:5(n-3). *TpLACS* has been characterized by heterologous expression in yeast by (Tonon et al., 2005a). In these studies, the enzyme was shown to convert externally supplied VLCPUFAs into the respective CoA esters with DHA as the preferred substrate (Tonon et al., 2005a). Thus, the rationale behind this co-expression experiment was to enhance 22:5(n-3)-CoA levels in yeast cells in order to test whether Old4p exhibits increased activity under these conditions. However, co-expression of *Old4* with *TpLACS* did not result in a higher desaturation efficiency of Old4p, as can be seen in Figure 19. Whereas the desaturation efficiency upon co-expression of *Old4* with pESC-LEU was 16 % in the experiment performed, co-expression with *TpLACS* led to a lower conversion rate of about 9 %. It could also be observed that the total amount of 22:5(n-3) in *TpLACS*-expressing cultures was not significantly higher compared to cultures expressing the empty vector.

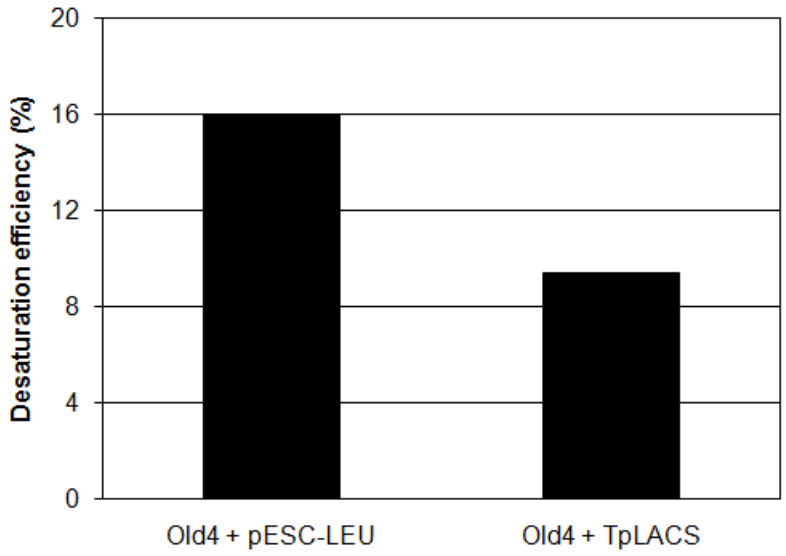

Figure 19. Conversion rates of 22:5(n-3) after co-expression of *Old4* with *TpLACS* in yeast

Old4-pESC-TRP was co-expressed with pESC-LEU or TpLACS-pESC-LEU for three days at 23 °C in INVSc1 cells supplemented with 200 µM 22:5(n-3). FAMEs from yeast cells were prepared and analyzed by GC-FID. Desaturation efficiency was calculated as 100*product/(substrate + product). Bars represent the result of one experiment.

Some chloroplastidic desaturases have been shown to act specifically on acyl chains that are linked to galactolipids like MGDG (Norman et al., 1991; Heilmann et al., 2004; Gao et al., 2009). This lipid is the most abundant chloroplastidic lipid and highly enriched in 16:3(n-3) as well as in 18:3(n-3) (Vijayan and Browse, 2002). Because yeast does not produce galactolipids endogenously, missing substrate in this form might be a reason for low desaturation rates of Old4p as well. To test this hypothesis, Old4p was introduced together with the MGDG synthase from *C. sativus* (CsMGDGS; (Shimojima et al., 1997)) into yeast. *CsMGDGS* has been successfully used in co-expression studies with chloroplastidic desaturases from *A. thaliana* in yeast before and led to a switch in the double bond positioning of the tested desaturases (Heilmann et al., 2004). Also in these experiments, MGDG was produced in yeast cultures expressing *CsMGDGS*, as can be seen in Figure 20 A. However, supplemented 22:5(n-3) was not incorporated into galactolipids (data not shown), most probably due to substrate restrictions of the cucumber enzyme. When 16:3(n-3) was supplemented in cultures expressing *CsMGDGS* and *Old4*, the fatty acid was subsequently found in the galactolipid fraction. In these cultures, nevertheless, no desaturation product was detectable. In cultures co-expressing *Old4* with pESC-LEU, desaturation rates with 1.4 % were comparable to those that were observed for expression of *Old4* alone (Figure 20 B and Figure 17).

In conclusion, Old4p desaturated substrates in a lipid-dependent manner and did not reveal a preference for MGDG-bound acyl chains.

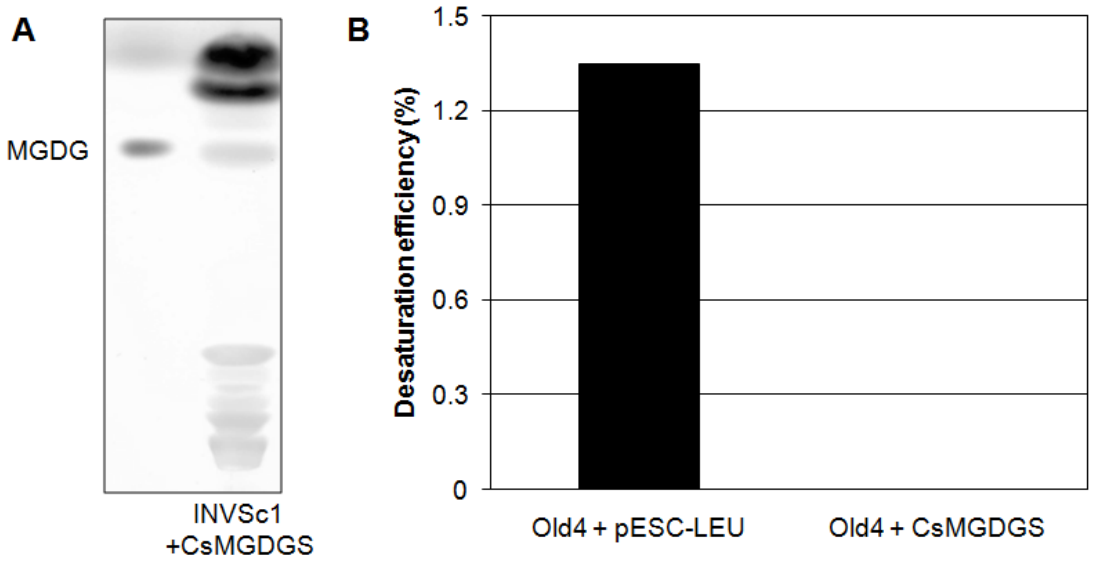

Figure 20. Results after expression of *MGDGS* and *Old4* in yeast

(A) TLC plate after expression of *CsMGDGS* for three days at 23 °C in INVSc1, subsequent lipid extraction and separation. (B) Conversion rates of 16:3(n-3) after co-expression of *Old4* with *CsMGDGS*. Old4-pESC-TRP was co-expressed with pESC-LEU or CsMGDGS-pESC-LEU for three days at 23 °C in INVSc1 cells supplemented with 200 µM 16:3(n-3). FAMEs from yeast cells were prepared and analyzed by GC-FID. Desaturation efficiency was calculated as 100*product/(substrate + product). MGDG, monogalactosyldiacylglycerol. Bars represent the mean of two independent experiments.

3.2.5 Comparative expression of Old4 and Egd4 and localization tests

To find out whether the rather weak desaturation activity of Old4p was due to low protein levels in yeast, Western Blot studies were performed to compare protein amounts accumulating after expression of *Old4* and *Egd4*, respectively. For these studies, both desaturase cDNAs were cloned into the pUG36 vector (by courtesy of Dr. R. Krick) in order to obtain aminoterminal GFP-tagged fusion proteins. pUG36 contains a negatively regulated methionine-dependent promoter that drives expression in absence of methionine. Yeast cultures expressing the constructs were grown for two days at 23 °C and under different methionine concentrations in order to test different expression levels. Afterwards, cultures were harvested and protein lysates were produced. These were then analyzed by Western Blot under usage of a GFP-specific antibody. Figure 21 shows similar levels of both desaturase fusion proteins after moderate expression (+ 0.3 mM Met) as well as after over-expression (- Met). To check whether mislocalization of the microalgal desaturase Old4p causes the low enzyme activity, yeast cells harbouring the GFP-fusion construct were examined under the microscope. Old4p did not form aggregates in yeast cells and seemed to localize to the ER, as can be seen in Figure 21. This reflected the expected localization of a typical microsomal front-end desaturase.

Taken together, these results suggested that neither low protein amounts nor enzyme mislocalization were diminishing factors for desaturase activity of Old4p.

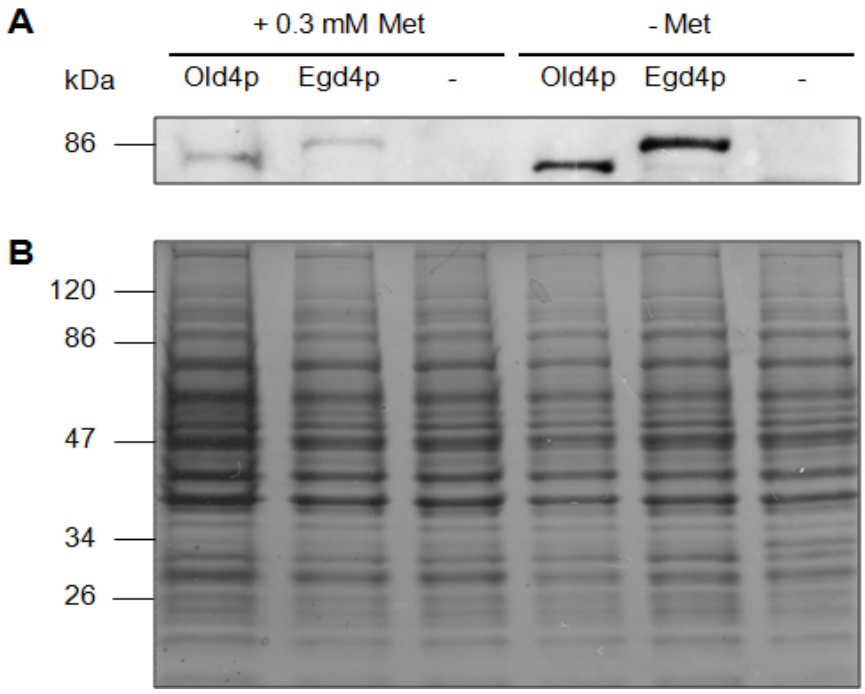

Figure 21. Protein amounts after moderate and strong expression of *GFP-Old4* and *GFP-Egd4*

GFP-Old4-pUG36, GFP-Egd4-pUG36 or pUG36 (-; negative control) were expressed for two days at 23 °C in INVSc1 either in presence of 0.3 mM methionine (+ 0.3 mM Met; moderate expression) or in complete absence of methionine (- Met; strong expression). Cells were lysed and total lysates were analyzed. (A) Western Blot and (B) corresponding Coomassie gel as loading control. GFP-Old4p is 78 kDa, GFP-Egd4p is 87 kDa in size. The experiment was repeated two times with similar results.

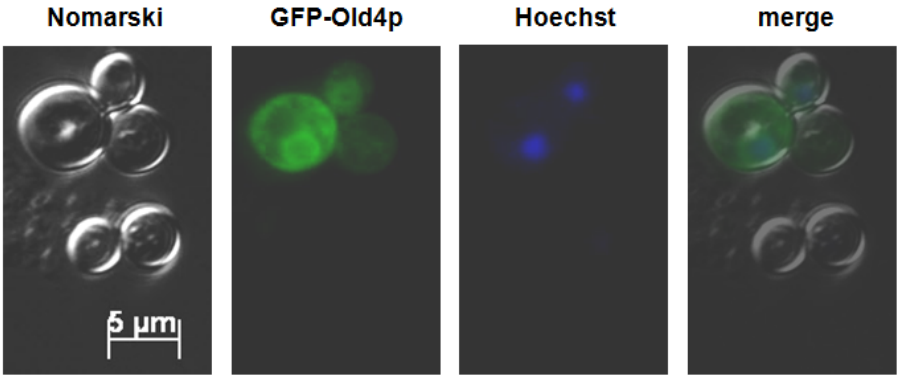

Figure 22. Localization of GFP-Old4p in yeast cells

INVSc1 cells expressing GFP-Old4-pUG36 were grown to stationary phase at 30 °C. Cell nuclei were stained and cells were examined under the microscope. Presented is a representative result of two independent experiments.

3.3 Characterization of acyltransferases, desaturases and elongases in plants

After establishing VLCPUFA production in plants, this process was investigated thoroughly and was extended by further enzymatic activities. This implicated the analysis of microalgal acyltransferases *in planta* as well as the new combination of already identified and characterized desaturases, elongases and acyltransferases, the transformation of the corresponding sequences into different plant species and subsequent analysis of transgenic seeds, sometimes also throughout consecutive generations.

3.3.1 Expression of microalgal acyltransferase sequences in *A. thaliana*

The previously analyzed microalgal acyltransferase sequences were expressed in *A. thaliana* in order to find out which effect they have on the seed lipid metabolism. Therefore, the partly or completely codon-optimized sequences of *OtDGAT2B*, *OlLPCAT*, *OtLPCAT* and *OtPDAT* were cloned behind the seed-specific *LeB4* promoter into pCAMBIA33.2cGs and subsequently transformed into *A. thaliana* plants. Alongside with the mentioned constructs, the empty vector was transformed as negative control into *Arabidopsis*. T1 seeds were harvested and Basta-selected. The derived T2 seeds then were analyzed with respect to their TAG content as well as to their fatty acid composition. Results from TAG content determinations are depicted in Figure 23. No significant changes could be observed upon expression of different acyltransferase sequences in seeds in comparison to the seeds expressing empty vector control.

Results from the determination of fatty acid composition of transgenic *Arabidopsis* seeds expressing different acyltransferase sequences are depicted in Figure 24 and Figure 25. Expression of *OtDGAT2B* did not change the fatty acid composition, as can be seen in Figure 24 A. Expression of *OtPDAT* led to minor changes, enhancing 20:1(n-9), 20:2 and 22:1 about 2.2 %, 0.3 % and 0.6 %, respectively. At the same time, levels of 18:0 and 18:1(n-7) were decreasing about 0.3 % and 0.7 % in comparison to those seeds expressing the empty vector control (Figure 24 B). In seeds expressing *OlLPCAT*, the strongest effects were observed (Figure 25 A). 18:0 and 18:1(n-9) amounts were decreasing about 0.3 % or 3.7 %, respectively. Concomitantly, 18:2(n-6) and 18:3(n-3) levels were increasing about 1.1 % or 2.8 % in comparison to the control. Further changes were observable for levels of 16:0 (increase of 0.8 %), 16:1 (increase of 0.11 %), 20:1(n-9) (decrease of 1.5 %) and 20:2 (increase of 0.4 %). Expression of *OtLPCAT* also led to a small increase in 18:3(n-3) of 1 %. At the same time, 20:1(n-7) amounts were 0.4 % larger and 20:0 amounts were decreasing about 0.2 % (Figure 25 B).

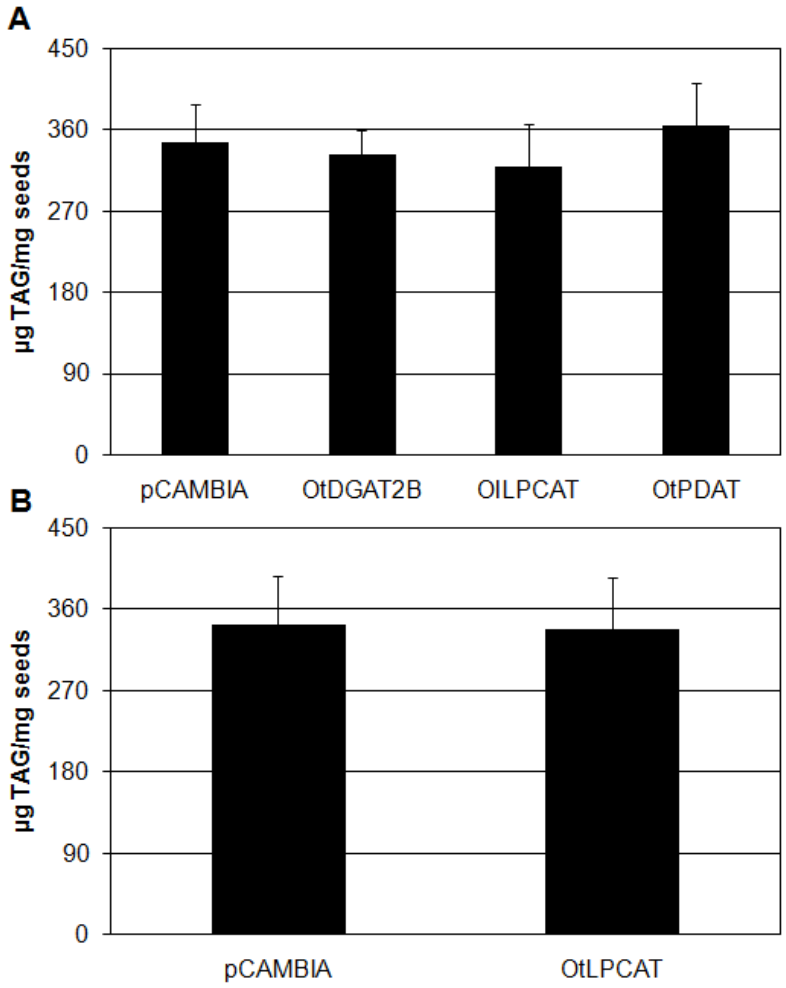

Figure 23. TAG content after expression of microalgal acyltransferase sequences in *Arabidopsis*

(A) TAG content of T2 seeds expressing pCAMBIA33.2cGs (negative control), OtDGAT2B-pCAMBIA33.2cGs, OlLPCAT-pCAMBIA33.2cGs or OtPDAT-pCAMBIA33.2cGs. (B) TAG content of seeds expressing pCAMBIA33.2cGs (negative control) or OtLPCAT-pCAMBIA33.2cGs after pool analysis of 3 mg seeds per plant line. TAG, triacylglycerol. Bars represent mean values + SD of ten to twenty independent plant lines.

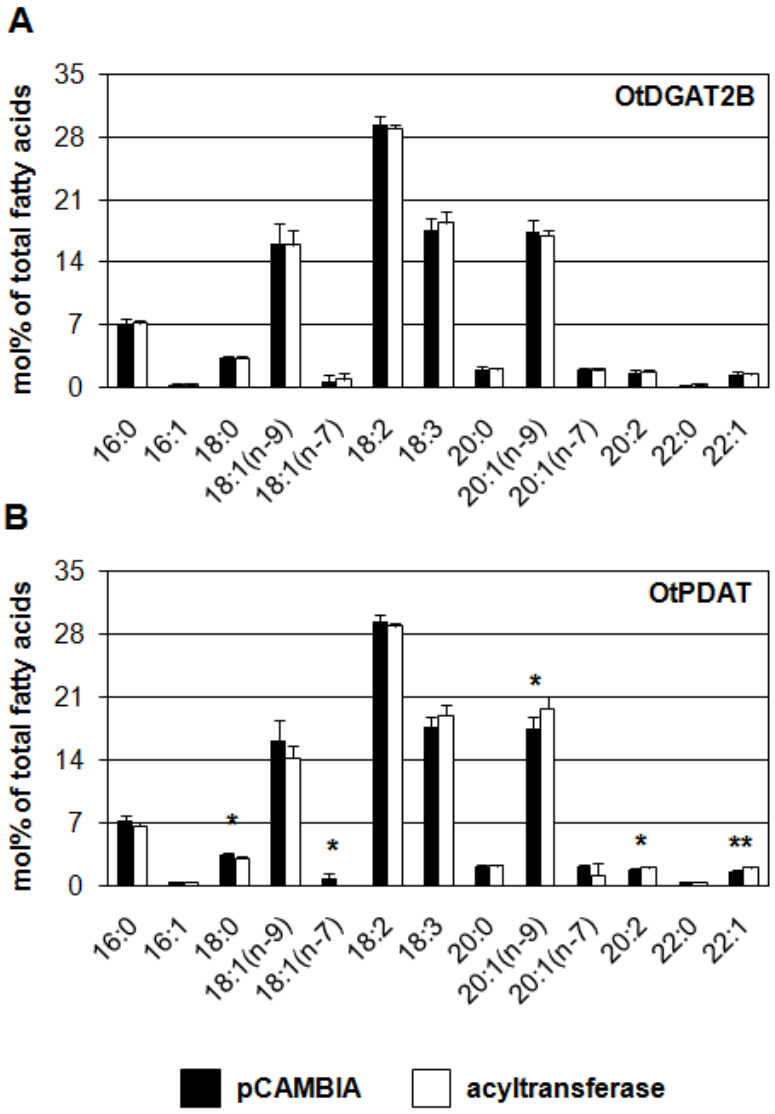

Figure 24. Fatty acid composition after expression of *OtDGAT2B* and *OtPDAT* in *Arabidopsis* seeds

Fatty acid composition of total lipids after pool analysis of 3 mg T2 seeds expressing pCAMBIA33.2cGs (negative control) compared to (A) OtDGAT2B-pCAMBIA33.2cGs or (B) OtPDAT-pCAMBIA33.2cGs. Bars represent mean values + SD of ten independent plant lines. *, $p < 0.01$ and **, $p < 0.001$ determined with Student's *t*-test.

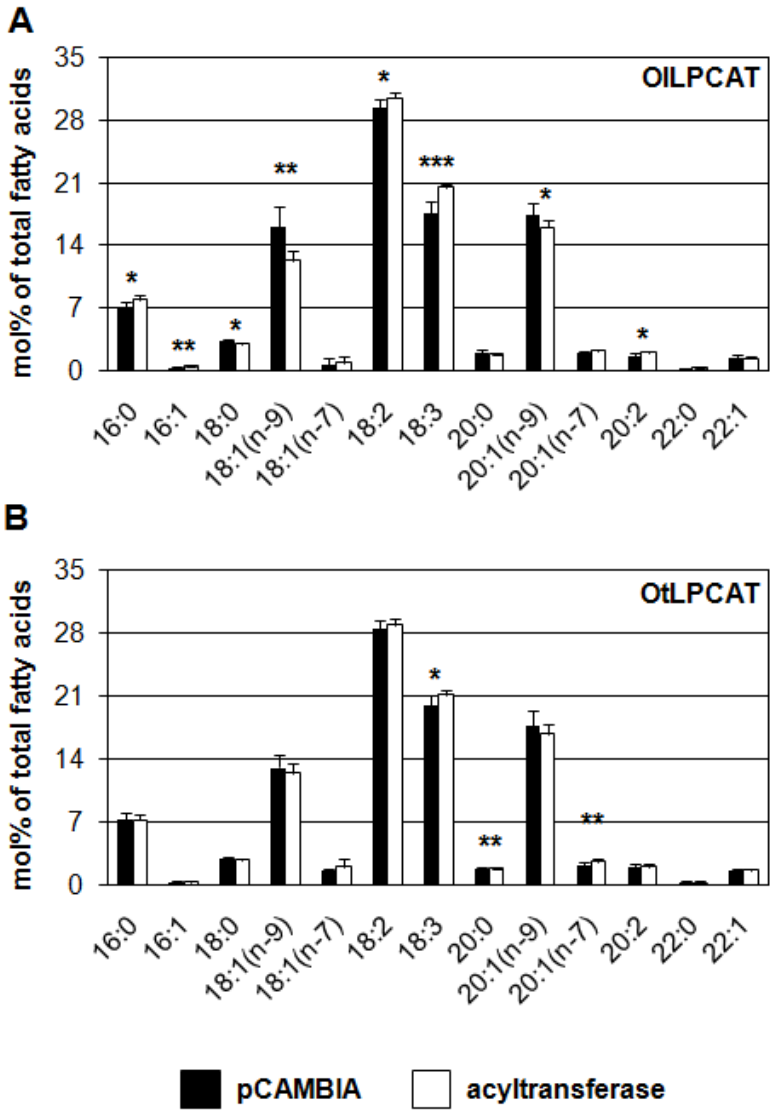

Figure 25. Fatty acid composition after expression of *OlLPCAT* and *OtLPCAT* in *Arabidopsis* seeds

Fatty acid composition of total lipids after pool analysis of 3 mg T2 seeds expressing pCAMBIA33.2cGs (negative control) compared to (A) OlLPCAT-pCAMBIA33.2cGs or (B) OtLPCAT-pCAMBIA33.2cGs. Bars represent mean values + SD of ten to twenty independent plant lines. *, $p < 0.01$ and **, $p < 0.001$ and ***, $p < 0.0001$ determined with Student's *t*-test.

Because *OlLPCAT* expression seemed to increase 18:3(n-3) amounts at the expense of 18:1(n-9) in total lipids, it was tested which lipid class is affected in transgenic seeds. For this purpose, total lipids were extracted and the most prominent lipid fractions in seeds, the TAG and the PC fraction, were analyzed. Fatty acid composition of both fractions was compared with those from control plants expressing empty vector. Results from these analyses are depicted in Figure 26. No differences in the fatty acid composition of PC between control seeds and OlLPCAT-expressing seeds could be observed (Figure 26 A). In the TAG fraction, however, an increase in 18:2(n-6) amounts of 1.3 % and in 18:3(n-3) amounts of 2 % was detectable. At the same time,

18:0 und 20:0 levels were decreasing about 0.5 % and 0.4 %, respectively (Figure 26 B). Because seeds expressing empty vector showed a high standard deviation in 18:1(n-9)-levels, no significant difference could be observed in comparison to seeds expressing *OlLPCAT*.

In summary, expression of *OlLPCAT* showed the strongest effects in *Arabidopsis* seeds by decreasing 18:1(n-9) levels and increasing amounts of polyunsaturated 18-carbon chain fatty acids. These changes could be pinpointed to the TAG fraction of seeds.

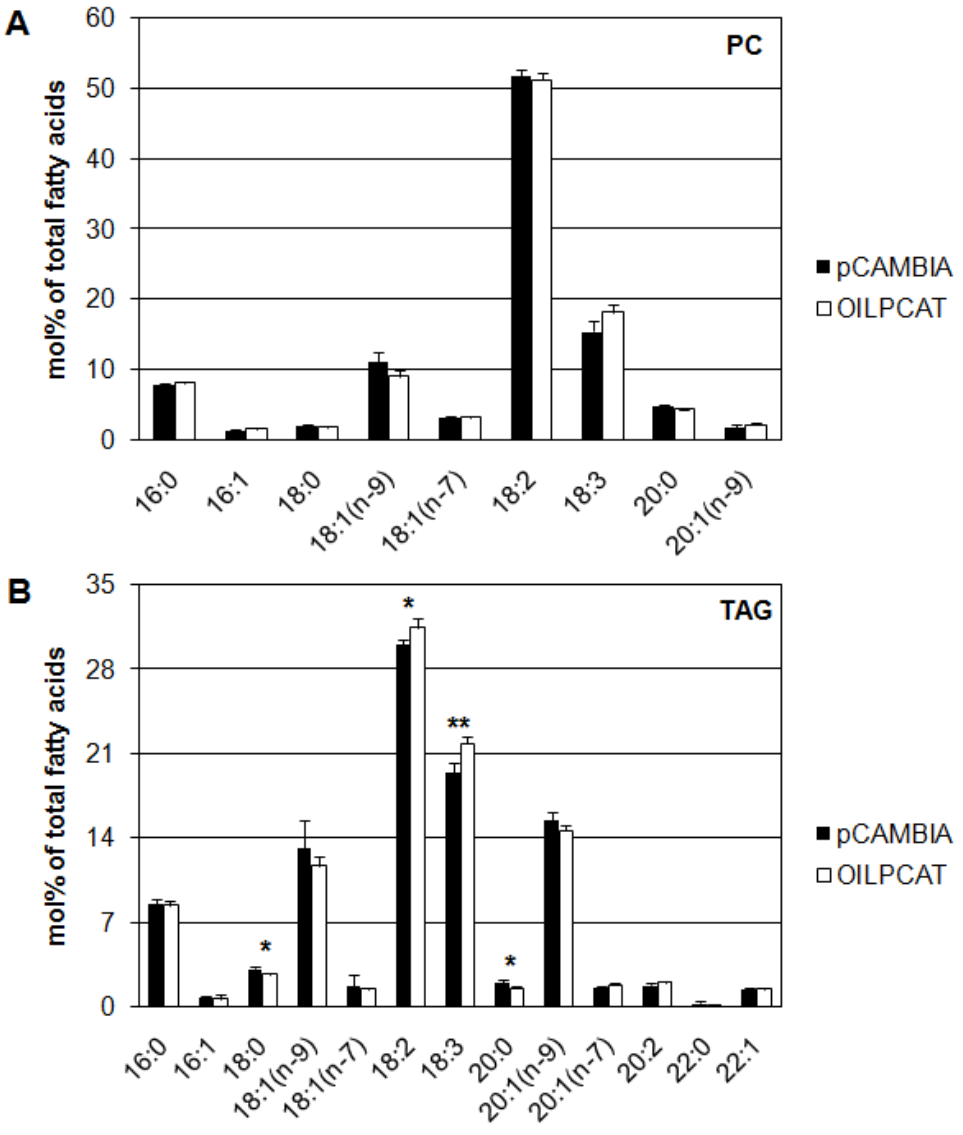

Figure 26. PC and TAG fatty acid compositions after expression of *OILPCAT* in *Arabidopsis*

Fatty acid composition of (A) PC and (B) TAG from 3 mg T2 seeds expressing pCAMBIA33.2cGs (negative control) compared to OILPCAT-pCAMBIA33.2cGs was determined by total lipid extraction, separation and GC analysis of different lipid fractions. PC, phosphatidylcholine; TAG, triacylglycerol. Bars represent mean values + SD of five independent plant lines. *, $p < 0.01$; **, $p < 0.001$ determined with Student's *t*-test.

3.3.2 Complementation experiments in the *A. thaliana tag1-1* mutant

The *Arabidopsis tag1-1* mutant, also known as AS11 mutant, has been identified in an ethane methyl sulfonate screen by Katavic and co-workers in 1995. It revealed much higer levels of ALA (18:3(n-3)) and diminished levels of oleic acid (18:1(n-9)) as well as eicosenoic acid (20:1(n-9)) in mature seeds (Katavic et al., 1995). In further studies, mutant seeds were shown to also have a strongly reduced TAG content in comparison to wild type seeds (Zou et al., 1999). The mutated gene was identified to encode a DGAT1p and was supposed to be a major factor influencing TAG content and composition in *Arabidopsis* seeds (Katavic et al., 1995; Routaboul et al., 1999; Zou et al., 1999).

Mutant seeds were analyzed side-by-side with wild type seeds in order to check whether effects that are described in literature could be reproduced under our experimental conditions. For this purpose, mutant and wild type plants were cultivated in the climate chamber and their total seed lipids were analyzed with respect to their TAG content and their fatty acid composition. Results of these measurements are depicted in Figure 27. Overall TAG content in mutant seeds was with 250 µg/mg seeds about 100 µg lower than TAG content of wild type seeds, as can be seen in Figure 27 A. Fatty acid composition of mutant seeds was also altered in comparison to wild type seeds with higher levels of 18:3(n-3) and lower levels of 18:1 and 20:1 (Figure 27 B). Taken together, these results confirmed the data obtained by other groups.

Next, it was tested whether expression of the microalgal *OtDGAT2B* is able to rescue the observed phenotype. Thus, the construct OtDGAT2B-pCAMBIA33.2cGs carrying the acyltransferase sequence downstream of the seed-specific *LeB4*-promoter was transformed into *tag1-1* mutant plants. As positive controls, *AtDGAT1* and *AtDGAT2* from *Arabidopsis* were brought into the same expression vector and were also transformed into the mutant. As negative control, empty vector was transformed into *tag1-1* plants. As further control, wild type *Arabidopsis* plants transformed with empty vector were used. T1 plants were cultivated in the greenhouse, Basta-selected and their T2 seeds were used for analyses of TAG content and fatty acid composition of total seed lipids. Results from TAG analyses are illustrated in Figure 28. Surprisingly, TAG amounts were not significantly different in transgenic wild type and mutant lines containing empty vector or expressing different DGAT sequences. Overall, TAG amounts were with about 250 µg TAG/mg seeds as low as in the *tag1-1* mutant seeds analyzed before (Figure 27 and Figure 28).

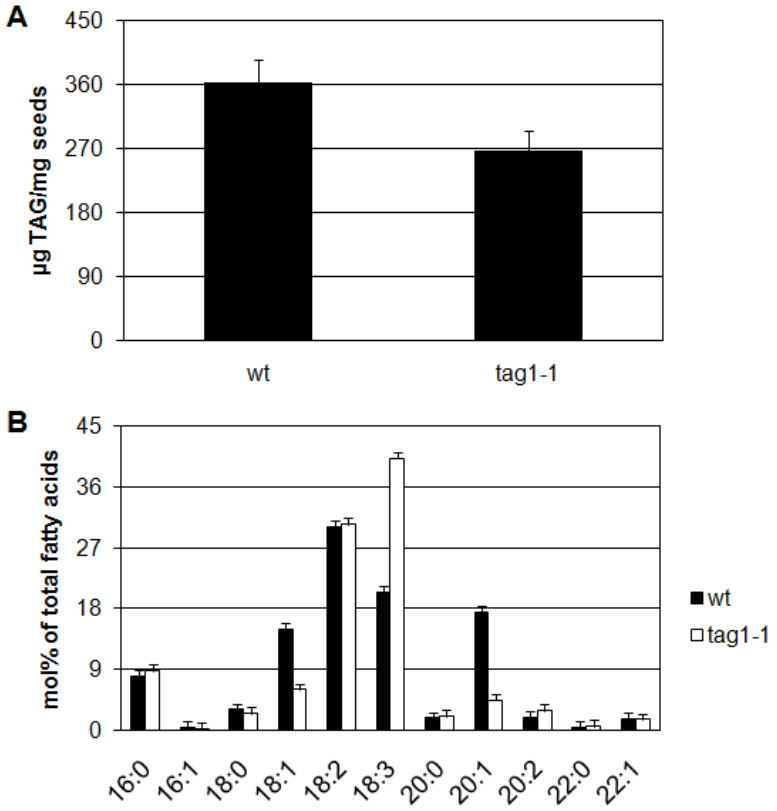

Figure 27. TAG content and fatty acid composition of *Arabidopsis* wt and *tag1-1* seeds

(A) TAG content and (B) fatty acid profile of Col-0 (wt) and *tag1-1* mutant seeds from pool analysis of 3 mg seeds per plant. TAG, triacylglycerol. Bars represent mean + SD of ten different plants.

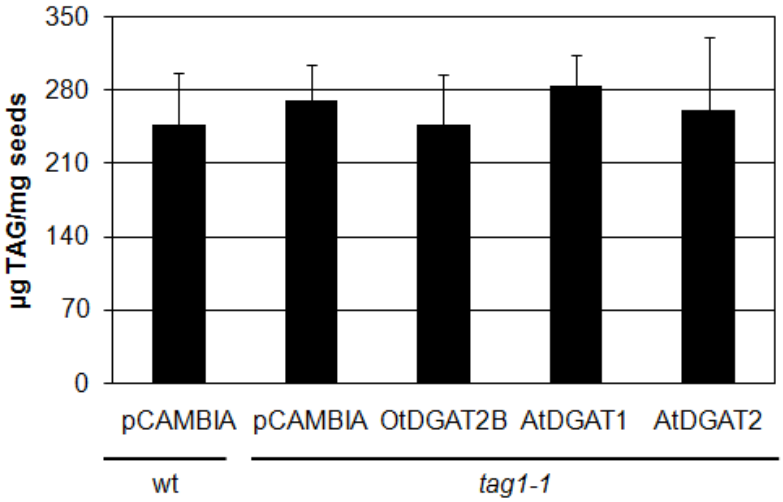

Figure 28. TAG content after expression of different DGAT sequences in *Arabidopsis* wild type and *tag1-1* mutant seeds

TAG content of 3 mg Col-0 (wt) or mutant T2 seeds expressing pCAMBIA33.2cGs, OtDGAT2B-pCAMBIA33.2cGs, AtDGAT1-pCAMBIA33.2cGs or AtDGAT2-pCAMBIA33.2cGs from pool analysis. TAG, triacylglycerol. Bars represent mean values + SD of ten different plants.

Results from the analyses concerning fatty acid composition of total seed lipids are depicted in Figure 29. In these measurements, the previously observed strong effects of the *DGAT1*-mutation could be confirmed. 18:3(n-3) levels were increased in *tag1-1* mutant seeds, whereas 18:1(n-9) and 20:1(n-9) levels were reduced in comparison to Col-0 wild type seeds. However, no significant differences between expression of empty vector, *OtDGAT2B*, *AtDGAT1* or *AtDGAT2* could be observed. Thus, it was not possible to rescue the mutant phenotype with the utilized DGAT constructs.

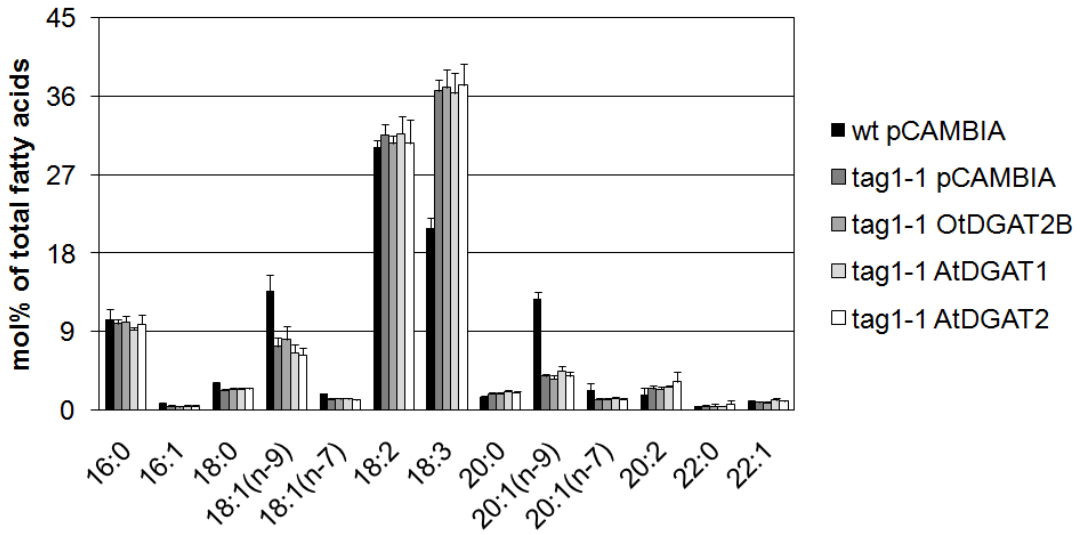

Figure 29. Fatty acid composition after expression of different DGAT sequences in *Arabidopsis* wild type and *tag1-1* mutant seeds

Fatty acid composition of total lipids of 3 mg wild type or mutant T2 seeds expressing pCAMBIA33.2cGs, OtDGAT2B-pCAMBIA33.2cGs, AtDGAT1-pCAMBIA33.2cGs or AtDGAT2-pCAMBIA33.2cGs. Bars represent mean values + SD of ten independent plant lines.

3.3.3 Combination of different desaturases with an elongase in *A. thaliana*

During previous studies, Dr. M. Heilmann isolated acyl-CoA-dependent desaturases from *O. tauri* (Δ5-desaturase Otd5p) and *M. squamata* (Δ5- and Δ6-desaturases Msd5p and Msd6p, respectively). Their corresponding cDNA sequences were co-expressed under the control of the unspecific seed protein (*USP*) promoter with the previously identified Δ6-desaturase sequence from *O. tauri* (*Otd6*, (Domergue et al., 2005)) and the moss elongase sequence *PSE1* (Zank et al., 2002) in the constructs Otd6-PSE1-Otd5-pCAMBIA3300 (Ot3) and Msd6-PSE1-Msd5-pCAMBIA3300 (Ms3) in *A. thaliana* to produce EPA in transgenic seeds. As control, the construct Ptd6-PSE1-Ptd5-pCAMBIA3300 (Pt3) containing, besides *PSE1*, the lipid-dependent *P. tricornutum* Δ6- and Δ5-desaturase sequences *Ptd6* and *Ptd5* described by (Abbadi et al., 2004) under control of the *USP* promoter, was also transformed into *Arabidopsis*. EPA amounts after expression of Ms3 and Pt3 were found to be about 0.03 %, EPA

amounts after expression of Ot3 were about 0.02 % of total fatty acids in the analyzed T2 seeds (Hoffmann et al., 2008). It was tested how the amounts of newly formed fatty acids alter in the consecutive seed generation. Therefore, transgenic T2 seeds from 22-30 Basta-selected T1 plants, representing independent transformation events for each expression construct, were analyzed. Two to four plant lines for each expression construct containing the highest amounts of (n-3)-PUFAs (sum from percentages of 18:4(n-3), 20:4(n-3) and 20:5(n-3) in total fatty acids) in their T2 seeds were then chosen for analysis in the T3 generation. Results of the screen are shown in Table 3. The percentage of (n-3)-PUFAs in T2 seeds expressing Ms3 was on average at 1.72 % showing a standard deviation of 1.27 %. Analysis of T3 seeds from different Ms3 T2 plant lines led to inconsistent results. Half of the examined lines showed higher amounts of (n-3)-PUFAs of 2.46 % and 2.51 %, respectively, in the next generation. The other T2 plant lines contained lower mean percentages of (n-3)-PUFAs of 1.12 % and 0.73 % in their T3 seeds. Also, the determined standard deviations were not declining. A similar trend could be observed also in case of (n-3)-PUFAs after Pt3 and Ot3 expression. The only exception in this respect constituted the (n-6)-PUFA percentages after expression of Pt3 which were on average at 3.53 % for T2 seeds, but at 5.15 % and 7.27 % for T3 seeds. Furthermore, determined standard deviations were quite high in these analyses. In conclusion, no uniform development of PUFA amounts in the course of different generations could be observed.

Table 3. Overview about (n-3)- and (n-6)-PUFA amounts in *Arabidopsis* seeds after expression of different VLCPUFA constructs

Pool analysis for 3 mg seeds per plant was performed. Ms3, Msd6-PSE1-Msd5-pCAMBIA3300; Pt3, Ptd6-PSE1-Ptd5-pCAMBIA3300; Ot3, Otd6-PSE1-Otd5-pCAMBIA3300. N indicates the number of tested plants. (n-3)-PUFA, sum from percentages of 18:4(n-3), 20:4(n-3) and 20:5(n-3) in total fatty acids; (n-6)-PUFA, sum from percentages of 18:3(n-6), 20:3(n-6) and 20:4(n-6) in total fatty acids. Mean values ± SD for percentages of newly formed fatty acids from total fatty acids are given.

construct	seed generation	N	(n-3)-PUFA (%)	(n-6)-PUFA (%)
Ms3	T2	22	1.72 ± 1.27	0.51 ± 0.60
Ot3	T2	28	1.94 ± 0.59	1.05 ± 0.67
Pt3	T2	30	1.88 ± 1.07	3.53 ± 4.05
Ms3	T3	30	2.46 ± 2.42	0.62 ± 0.74
Ms3	T3	29	2.51 ± 1.68	0.26 ± 0.38
Ms3	T3	28	1.12 ± 0.50	0.01 ± 0.05
Ms3	T3	29	0.73 ± 0.33	0.02 ± 0.10
Ot3	T3	29	1.18 ± 0.43	0.80 ± 0.21
Ot3	T3	31	2.16 ± 0.40	1.56 ± 0.46
Ot3	T3	30	1.63 ± 1.07	1.15 ± 0.95
Pt3	T3	29	1.29 ± 0.28	5.15 ± 1.09
Pt3	T3	30	2.09 ± 1.15	7.27 ± 5.70

3.3.4 Combination of acyl-CoA- and lipid-dependent desaturases in *A. thaliana*

The combination of lipid-dependent desaturases with an acyl-CoA-dependent elongase has been shown to lead to the accumulation of intermediates after the first desaturation step in linseed and tobacco. A possible reason for this is the insufficient transfer of substrates between PC pool and acyl-CoA pool (Abbadi et al., 2004). In subsequent studies, the utilization of acyl-CoA-dependent desaturases was shown to enhance substrate flux through the metabolic pathway of VLCPUFA production in plants. The only remaining problem was the low activity of the identified acyl-CoA-dependent desaturases with Δ5-specificity (Hoffmann et al., 2008). The following experiment was performed to investigate, whether the combination of the acyl-CoA-dependent desaturase Msd6p with the lipid-dependent desaturases Ptd6p and Ptd5p together with elongase PSE1p leads to higher EPA levels in seeds compared to the previously tested combinations. Thus, *Msd6* was cloned under the control of the seed-specific *USP* promoter into Ptd6-PSE1-Ptd5-pCAMBIA3300 (Pt3) and the resulting construct was transformed into *Arabidopsis*. T2 seeds of 13 Basta-selected independent T1 plant lines were screened for presence of newly produced fatty acids of the VLCPUFA pathways. Unfortunately, none of the tested lines revealed VLCPUFAs (data not shown). In a second approach, also the OlLPCATp from *O. lucimarinus* was combined with the lipid-dependent desaturase and PSE1p under the control of the *USP* promoter to check whether the putative acyltransferase is able to transfer intermediates of the pathway between the different acyl pools in seeds and thereby circumvents the described bottleneck. *OlLPCAT* hence was cloned into Pt3 and the resulting construct was transformed into *A. thaliana*. T2 seeds of 30 independent T1 plant lines were screened, but identified VLCPUFA amounts were much lower compared to Pt3 plants (data not shown). In a third approach, Pt3 was combined with both *Msd6* and *OlLPCAT* in order to find out whether co-expression leads to higher VLCPUFA yields in transgenic *Arabidopsis* seeds. Thus, both sequences were cloned into Pt3 and the resulting construct was transformed into plants. Results of the co-expression compared to beforehand detected (n-3)-PUFA levels (Hoffmann et al., 2008) are depicted in Figure 30. The first intermediate of the pathway, 18:4(n-3), was accumulating with 2 % at much higher levels in seeds expressing *OlLPCAT-Msd6*-Pt3 compared to seeds expressing Pt3 (0.7 %) or Ms3 (0.1 %). 20:4(n-3) amounts were with 0.9 - 1 % comparable in seeds expressing *OlLPCAT-Msd6*-Pt3 or Ms3. However, EPA yields after expression of *OlLPCAT-Msd6*-Pt3 with 0.3 % were much higher than after expression of Pt3 or Ms3, that both led to EPA amounts of 0.05 %. Generally, one should keep in mind that plants were cultivated at different times and product levels might be influenced by several other factors apart from the utilized enzymatic activities.

In conclusion, co-expression of *OlLPCAT* with *Msd6*, *Ptd6*, *PSE1* and *Ptd5* led to much higher EPA levels in *Arabidopsis* seeds compared to the constructs tested in previous studies.

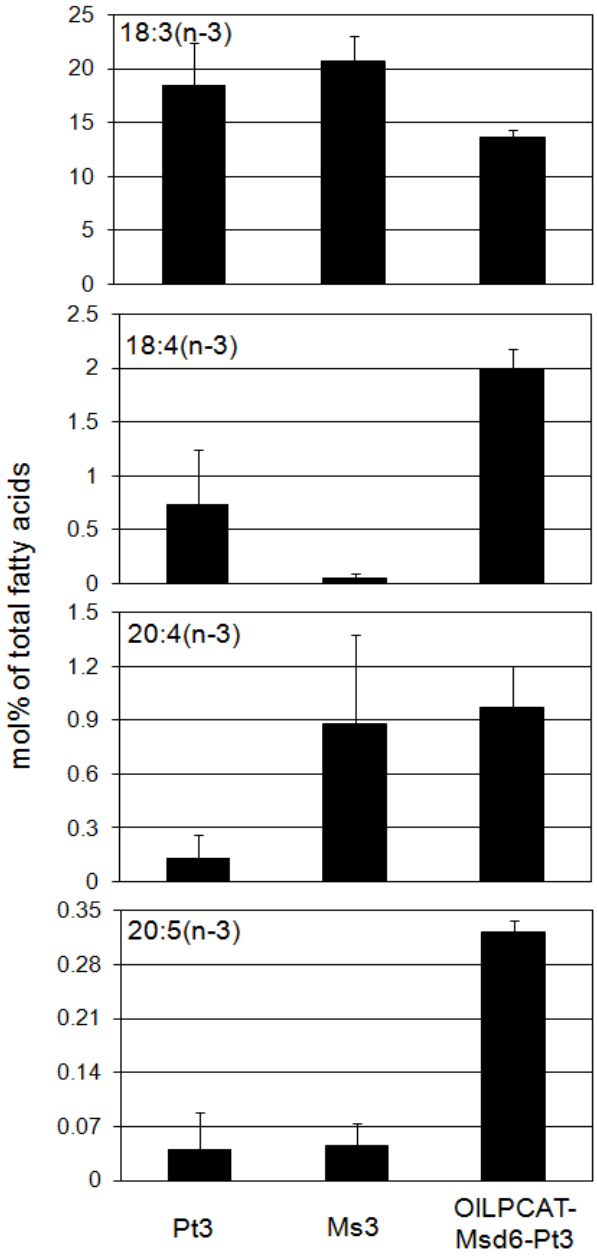

Figure 30. (n-3)-PUFA amounts in *Arabidopsis* seeds after expression of different VLCPUFA constructs

Pt3, Ptd6-PSE1-Ptd5-pCAMBIA3300; Ms3, Msd6-PSE1-Msd5-pCAMBIA3300. Pool analysis of 3 mg seeds per plant line was performed. Values from Pt3 and Ms3 plants are taken from (Hoffmann et al., 2008). Bars represent mean + SD of three independent plant lines.

3.3.5 Comparison of different host plants for VLCPUFA biosynthesis

For VLCPUFA production, various plant species like for example tobacco, linseed, soy, oilseed rape, *Arabidopsis*, Indian or Ethiopian mustard have already been utilized as host organisms (Abbadi et al., 2004; Qi et al., 2004; Robert et al., 2005; Wu et al., 2005; Hoffmann et al., 2008; Cheng et al., 2010). From these studies it could be learned that the choice of the host plants can have a great influence on product yields (Cheng et al., 2010). So far, our VLCPUFA constructs were tested solely in *Arabidopsis*. In order to figure out whether product yields can be increased by utilization of another, more suitable host plant, comparative studies between transgenic *Camelina sativa* and *A. thaliana* plants were performed.

C. sativa is an ancient oilseed crop. It is a member of the *Brassiceae* family and has been grown extensively in the nineteenth century (Knorzer, 1978). For unknown reasons, cultivation of *Camelina* diminished after the Second World War. However, in recent times this plant species has come back into focus of research due to its health-promoting fatty acid composition (Lu and Kang, 2008). In Figure 31, fatty acid profiles of *A. thaliana* and *C. sativa* wild type seeds are displayed. *Camelina* seeds are very rich in 18:3(n-3), resulting in a high (n-3) over (n-6) ratio. *Arabidopsis* seeds in contrast have a fatty acid composition that is typical for popular oilseed crops like oilseed rape or soybean. Their most prominent fatty acid is the (n-6)-fatty acid 18:2(n-6). That is why their (n-3) over (n-6) ratio is rather low.

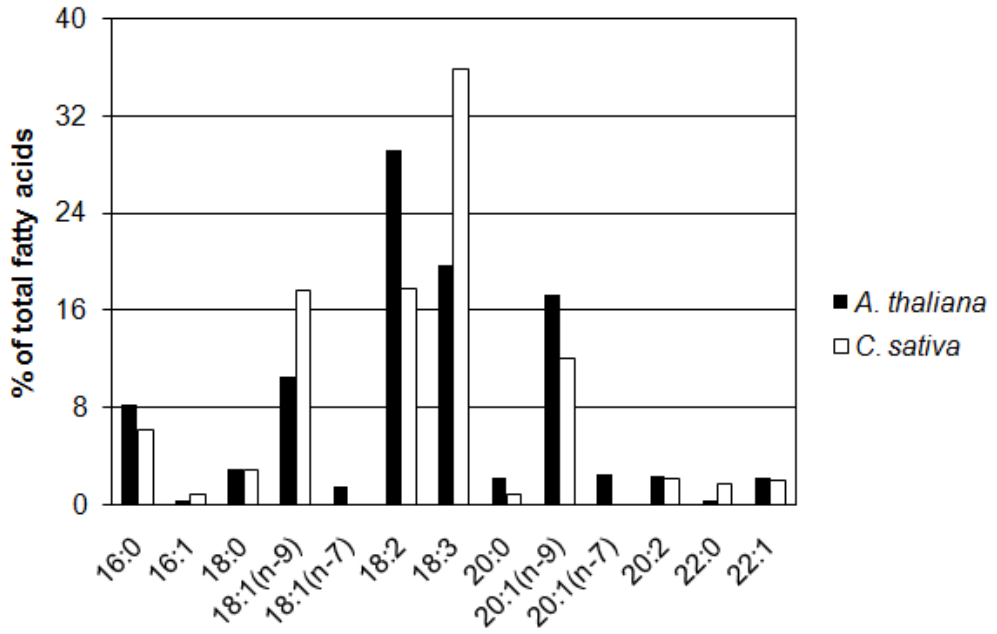

Figure 31. Fatty acid composition of *A. thaliana* and *C. sativa* wild type seeds

For *C. sativa*, single wild type seeds were analyzed, for *A. thaliana*, seed pool analysis of 3 mg seeds per plant was performed. Bars represent mean values from two independent samples.

Based on these observations, *C. sativa* was regarded as suitable production system for (n-3)-PUFAs because of its high initial substrate amounts. Therefore, flowering *Camelina* plants were transformed with Ptd6-PSE1-Ptd5-pCAMBIA3300 (Pt3) or the empty vector control based on the method developed by (Lu and Kang, 2008). T1 seeds were harvested and Basta-selected. Work concerning *Camelina* plants was done in cooperation with V. Behnen. T2 seeds were analyzed subsequently by single seed analysis and results were compared to Pt3-transformed T2 *Arabidopsis* seeds. T2 seeds were sown again. After a second selection round, T3 seeds were analyzed and also compared to transgenic *Arabidopsis* T3 seeds. Results of these comparative studies are illustrated in Figure 32 and Figure 33. As can be seen in Figure 32 A and B, *Arabidopsis* seeds in the T2 generation generally showed higher amounts of newly formed fatty acids of both (n-3)- and (n-6)-pathways compared to *Camelina* seeds. Solely, 20:4(n-6) amounts with 1.25 % were higher in *Camelina* seeds of the T2 generation. In contrast, amounts of newly formed (VLC)PUFAs were much lower in the analyzed T3 *Arabidopsis* seeds and rising *vice versa* in T3 *Camelina* seeds (Figure 33 A and B). Amounts of both (n-6)- and (n-3)-fatty acids in this generation were higher than in *Arabidopsis* seeds, reaching 20:4(n-6) yields of 4.2 % and EPA yields of 0.4 % of total fatty acids, respectively, in *Camelina.* It should be noted that transgenic *Camelina* T3 seeds looked shriveled, and their 18:3(n-3) amounts were about 18 % lower than in the analyzed wild type and transgenic T2 seeds (Figure 33 B, Figure 31 and Figure 32 B). 18:2(n-6) levels were comparable in all transgenic and wild type *Camelina* seeds (Figure 32 A, Figure 33 A and Figure 31). The precursor fatty acid 18:1(n-9) with 9-12 % was present at lower levels in transgenic *Camelina* seeds compared to wild type seeds (Figure 31).

In summary, expression of Pt3 in the two different host organisms *Arabidopsi*s and *Camelina* led to different product amounts, also revealing differences between the analyzed consecutive generations.

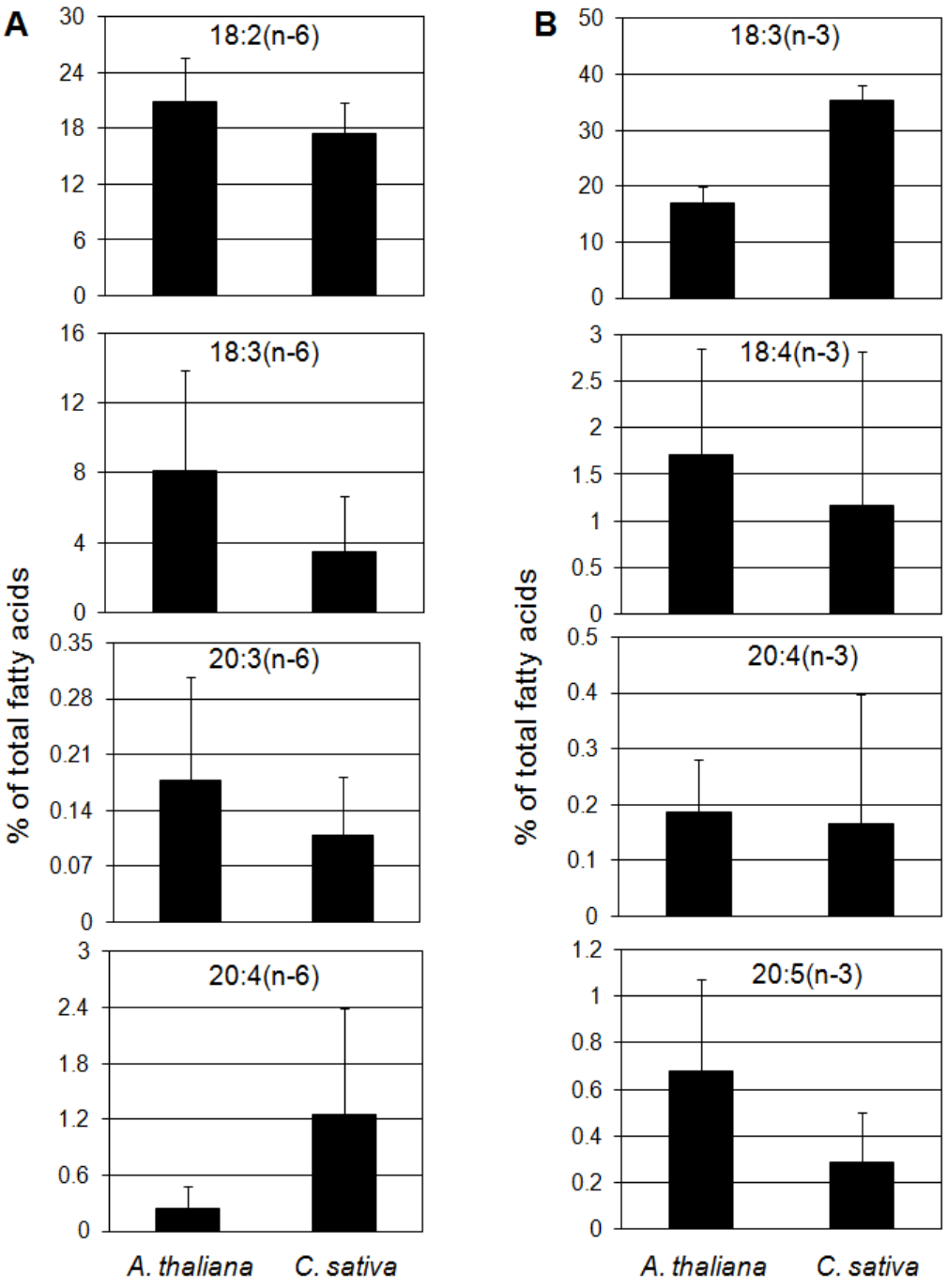

Figure 32. (n-6)- and (n-3)-PUFA contents of transgenic *A. thaliana* and *C. sativa* T2 seeds

Plants were transformed with Ptd6-PSE1-Ptd5-pCAMBIA3300, subsequently transgenic *A. thaliana* and *C. sativa* T2 seeds were analyzed by single seed analysis. (A) (n-6)-PUFA values and (B) (n-3)-PUFA values. Bars represent mean + SD from ten independent samples. Transformation of *Camelina* plants was performed in cooperation with V. Behnen.

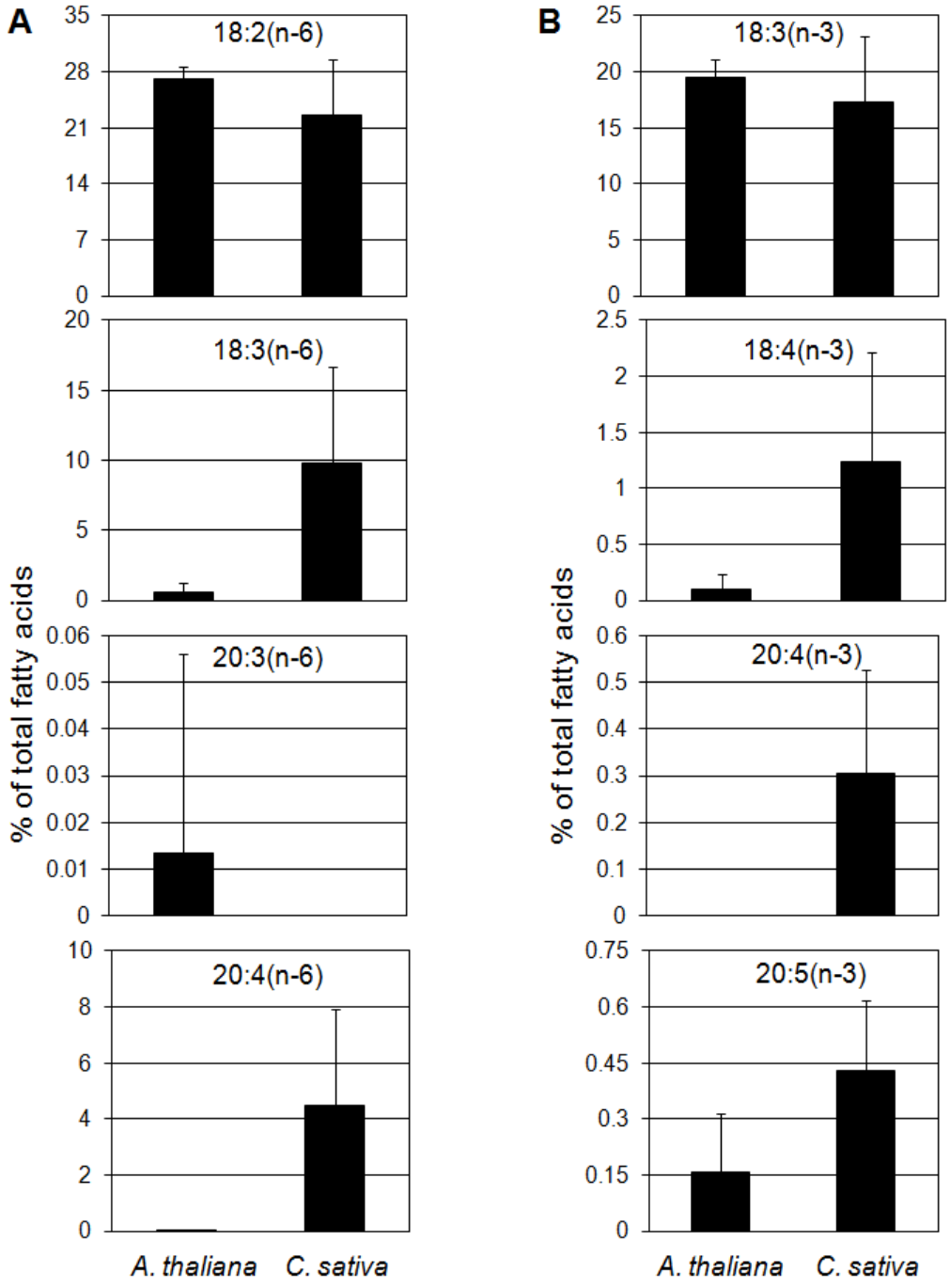

Figure 33. (n-6)- and (n-3)-PUFA contents of transgenic *A. thaliana* and *C. sativa* T3 seeds

Plants were transformed with Ptd6-PSE1-Ptd5-pCAMBIA3300, subsequently transgenic *A. thaliana* and *C. sativa* T3 seeds were analyzed by single seed analysis. (A) (n-6)-PUFA values and (B) (n-3)-PUFA values. Bars represent mean + SD from ten independent samples. Transformation of *Camelina* plants was performed in cooperation with V. Behnen.

3.3.6 Combination of microalgal acyltransferases with desaturases and an elongase in *A. thaliana*

After the separated analysis of transgenic seeds expressing acyltransferase sequences or sequences encoding VLCPUFA-producing enzymes, the next step was to investigate the interplay between desaturases, elongases and acyltransferases upon combination *in planta*. Therefore, Ptd6-PSE1-Ptd5-pCAMBIA2200 (Pt3, (Abbadi et al., 2004)) was transformed in one step alongside with the different acyltransferase-pCAMBIA33.2cGs constructs into *Arabidopsis* plants. T1 seeds were first selected on 1/2 MS medium containing kanamycin to screen for Pt3-positive plants. Subsequently, surviving seedlings were put on soil and were sprayed with Basta to select for acyltransferase-expressing plants. Surviving plants after this second selection were then cultivated and their T2 seeds were used for analysis of TAG content and VLCPUFA yields. Due to two independently transformed constructs and thus two necessary consecutive selection rounds, only few plants survived and were available for analysis. Results from the TAG analyses are shown in Figure 34. No significant differences could be observed in the TAG levels between those seeds co-expressing the empty vector with Pt3 and those expressing *OtDGAT2B*, *OtPDAT*, *OlLPCAT* or *OtLPCAT* together with Pt3. Mean VLCPUFA yields in plant lines co-expressing Pt3 with different acyltransferase sequences are depicted in Figure 35 and Figure 36. In general, both (n-6)- and (n-3)-VLCPUFA levels were similar in all analyzed plant lines and standard deviations were quite high. Expression of the empty vector together with Pt3 resulted in almost all cases to slightly higher VLCPUFA amounts compared to the co-expression with acyltransferase sequences. In conclusion, co-expression with different acyltransferase sequences did not improve the yield of VLCPUFA production by Pt3 expression.

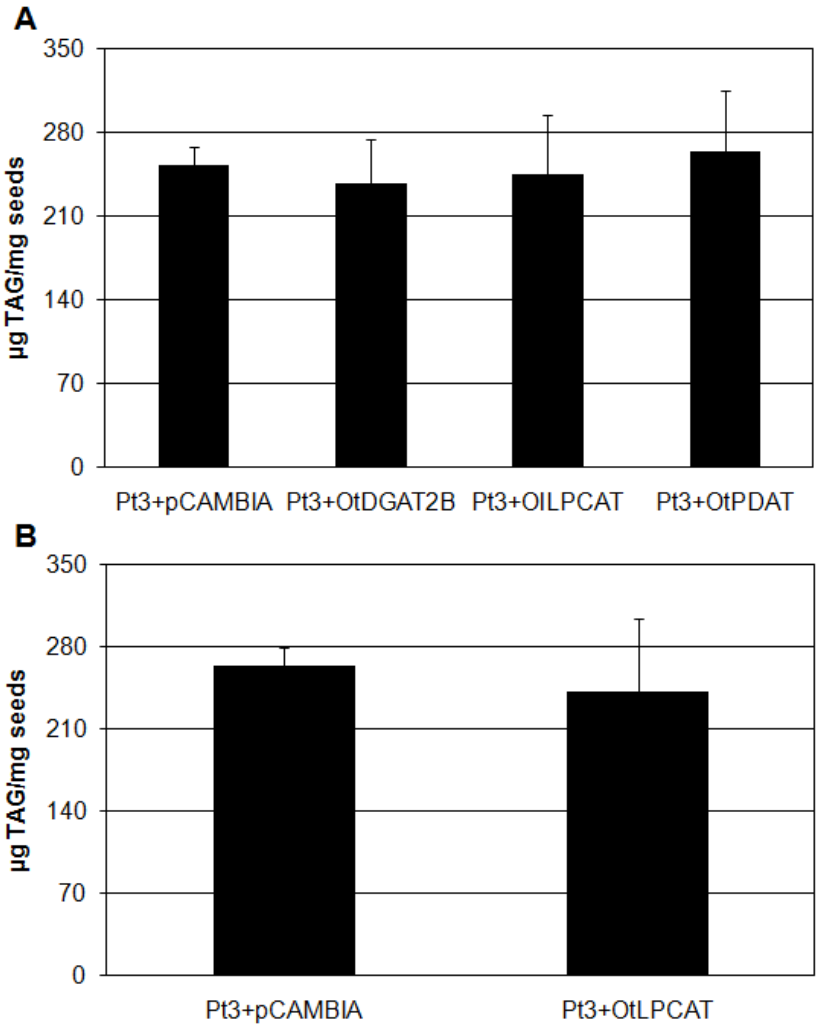

Figure 34. TAG content after co-expression of microalgal acyltransferase sequences with Pt3 in *Arabidopsis*

(A) TAG content of 3 mg T2 seeds co-expressing Ptd6-PSE1-Ptd5-pCAMBIA2200 (Pt3) with pCAMBIA33.2cGs, OtDGAT2B-pCAMBIA33.2cGs, OlLPCAT-pCAMBIA33.2cGs or OtPDAT-pCAMBIA33.2cGs. (B) TAG content of 3 mg T2 seeds co-expressing Pt3 with pCAMBIA33.2cGs or OtLPCAT-pCAMBIA33.2cGs. TAG, triacylglycerol. Bars represent mean values + SD of three to eleven independent plant lines.

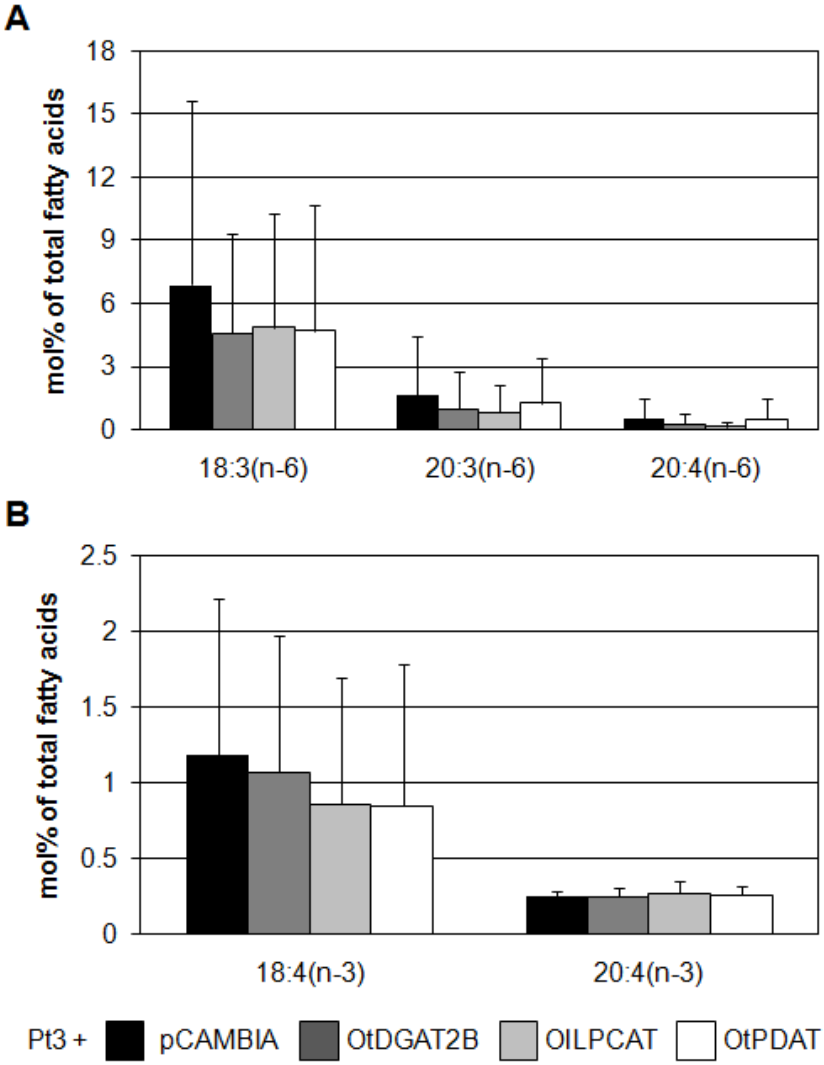

Figure 35. (n-6)- and (n-3)-PUFA contents of transgenic *A. thaliana* seeds co-expressing Pt3 with *OtDGAT2B*, *OILPCAT* or *OtPDAT*

Plants were co-transformed with Ptd6-PSE1-Ptd5-pCAMBIA2200 (Pt3) and pCAMBIA33.2cGs, OtDGAT2B-pCAMBIA33.2cGs, OlLPCAT-pCAMBIA33.2cGs or OtPDAT-pCAMBIA33.2cGs. 3 mg T2 seeds were analyzed by pool analysis. (A) (n-6)-PUFA values and (B) (n-3)-PUFA values. Bars represent mean + SD from three to eleven independent plant lines.

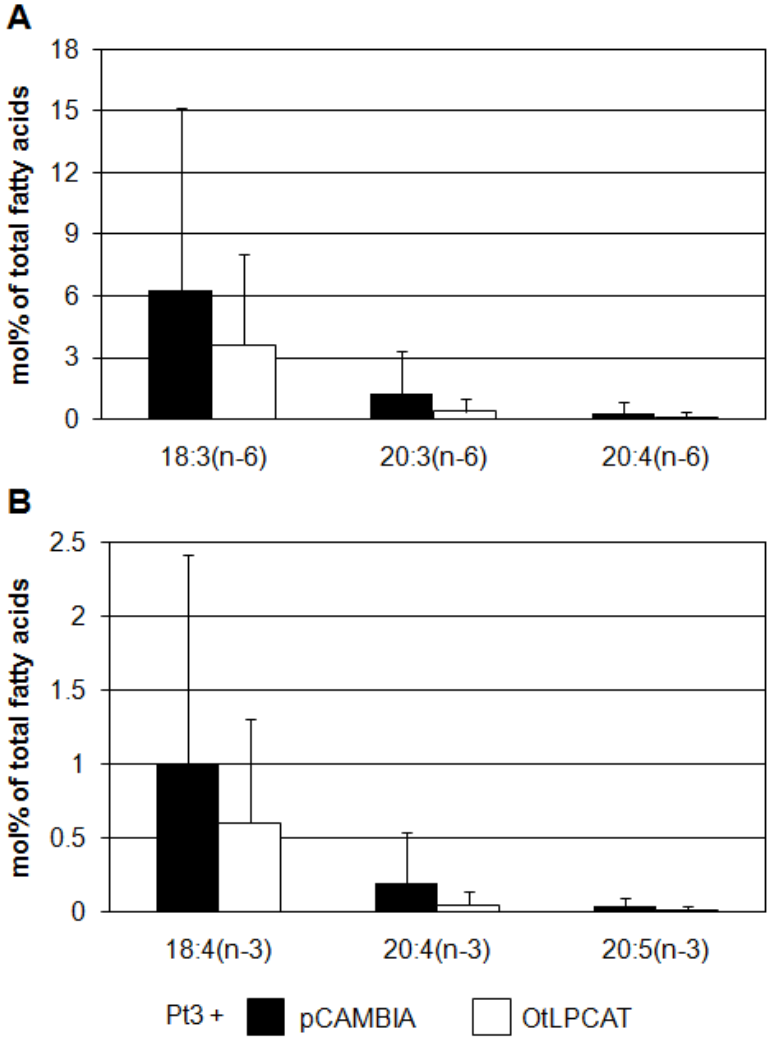

Figure 36. (n-6)- and (n-3)-PUFA contents of transgenic *A. thaliana* seeds co-expressing Pt3 with *OtLPCAT*

Plants were co-transformed with Ptd6-PSE1-Ptd5-pCAMBIA2200 (Pt3) and pCAMBIA33.2cGs or OtLPCAT-pCAMBIA33.2cGs. 3 mg T2 seeds were analyzed by pool analysis. (A) (n-6)-PUFA values and (B) (n-3)-PUFA values. Bars represent mean + SD from three to eleven independent plant lines.

3.3.7 Combination of microalgal acyltransferases with desaturases and an elongase in the *A. thaliana tag1-1* mutant

It was also tested whether VLCPUFA levels are higher in the *tag1-1* mutant upon expression of Ptd6-PSE1-Ptd5-pCAMBIA2200 (Pt3, (Abbadi et al., 2004)) and which effects co-expression of plant and microalgal DGAT sequences might have. Therefore, Pt3 was co-transformed with the different DGAT-pCAMBIA33.2cGs constructs containing either *AtDGAT1* or *OtDGAT2B* into *tag1-1* plants. T1 seeds were selected for presence of both constructs. Surviving plants were then cultivated in the greenhouse and their T2 seeds were used for analysis of TAG content and VLCPUFA yields. Results from the TAG analyses are shown in Figure 37. The overall TAG levels again were with 200-250 µg TAG/mg seeds as low as those from the non-transformed *tag1-1* seeds (Figure 27), but no differences could be observed between the different transgenic lines. Results from the analyses of VLCPUFA yields are illustrated in Figure 38. In the transgenic *tag1-1* mutant seeds much lower levels of (n-6)-PUFAs could be detected compared to the beforehand analyzed transgenic wild type seeds (Figure 36). In those mutant seeds expressing empty vector together with Pt3, 20:4(n-6) was not observable at all (Figure 38 A). In seeds co-expressing *AtDGAT1* or *OtDGAT2B* with Pt3, at least low 20:4(n-6) levels of about 0.2 mol% of total fatty acids were found. Surprisingly, also (n-3)-PUFA values were lower in comparison to wild type seeds, although (n-3)-substrate levels should be higher in the mutant seeds. For seeds co-expressing empty vector with Pt3, only 18:4(n-3) was detectable, but no 20:4(n-3) or EPA. However, it should be noted that the number of analyzed independent plant lines was rather low due to the double selection process and standard deviations were quite high.

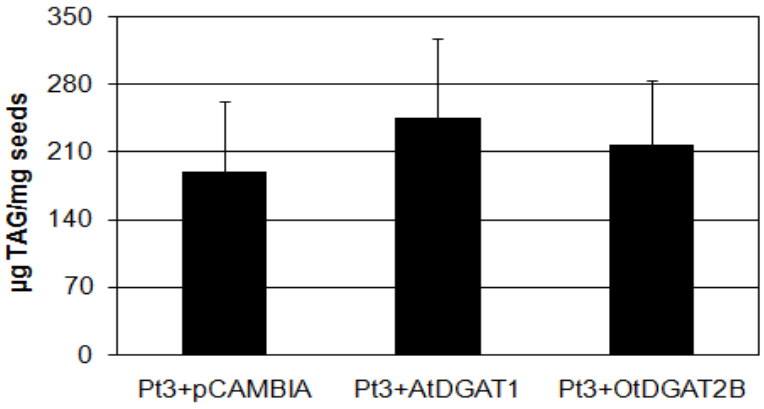

Figure 37. TAG content after co-expression of different acyltransferase sequences with Pt3 in *Arabidopsis tag1-1* seeds

TAG content from pool analysis of 3 mg T2 seeds co-expressing Ptd6-PSE1-Ptd5-pCAMBIA2200 (Pt3) with pCAMBIA33.2cGs, AtDGAT1-pCAMBIA33.2cGs or OtDGAT2B-pCAMBIA33.2cGs. TAG, triacylglycerol. Bars represent mean values + SD of four to six independent plant lines.

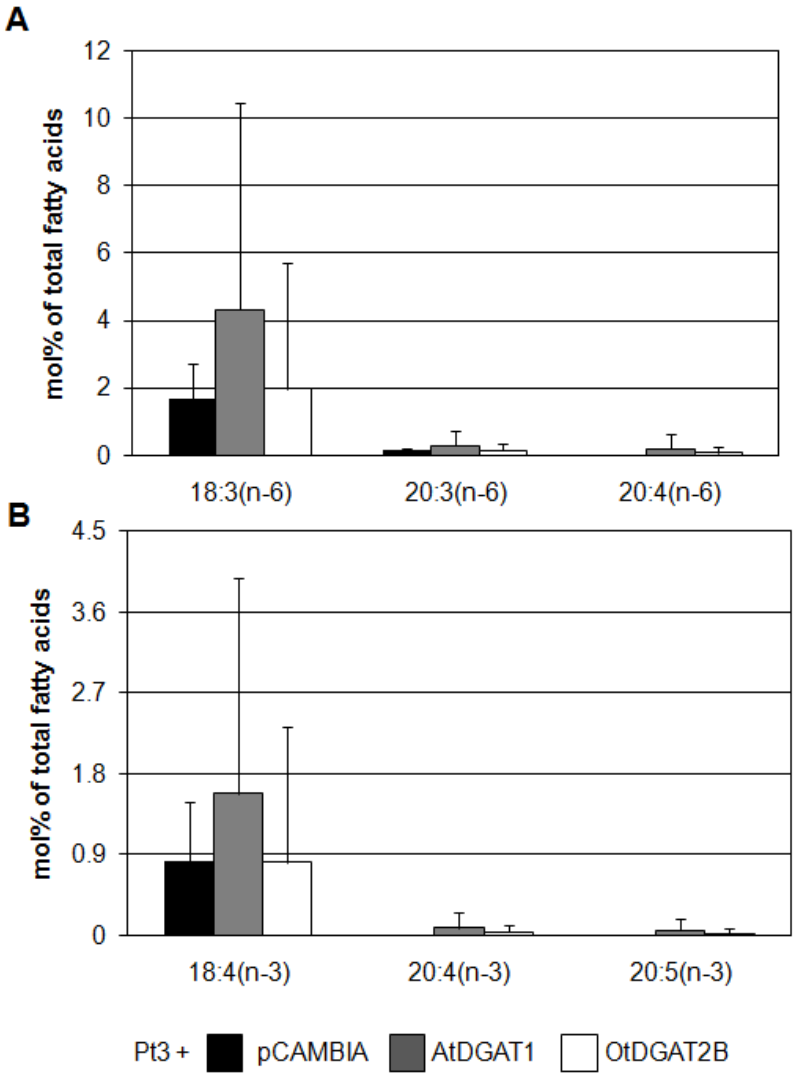

Figure 38. (n-6)- and (n-3)-PUFA contents of transgenic *A. thaliana tag1-1* seeds co-expressing Pt3 with *AtDGAT1* or *OtDGAT2B*

Plants were co-transformed with Ptd6-PSE1-Ptd5-pCAMBIA2200 (Pt3) and pCAMBIA33.2cGs, AtDGAT1-pCAMBIA33.2cGs or OtDGAT2B-pCAMBIA33.2cGs. 3 mg T2 seeds were analyzed by pool analysis. (A) (n-6)- PUFA values and (B) (n-3)-PUFA values. Bars represent mean + SD from four to six independent plant lines.

4 Discussion

4.1 Characterization of microalgal acyltransferases in yeast

Several nucleotide sequences encoding enzymes putatively involved in VLCPUFA distribution between different lipid fractions were isolated from *O. lucimarinus* and *O. tauri*. Most of the corresponding proteins featured acyltransferase activity upon expression in yeast (Wagner, 2008). The aim of this work was to further investigate their substrate specificities by heterologous expression studies in yeast and to find out whether they are useful candidates to change the fatty acid composition of seed oil.

4.1.1 OlLPCATp and OtLPCATp do not reveal LPLAT activity *in vitro*

In a previous study, expression of *OlLPCAT* from *O. lucimarinus* complemented a yeast mutant deficient in intrinsic LPCAT activity (Wagner, 2008) and was thus a promising candidate for further experiments regarding its substrate specificity. The closely related nucleotide sequence *OtLPCAT* was isolated recently by Dr. M. Heilmann from *O. tauri* and was also chosen for further investigations. Yeast has been successfully used as heterologous expression host already for the characterization of two LPLAT enzymes from *Arabidopsis*. Upon expression in yeast, both plant proteins revealed broad LPLAT activity *in vitro* (Stahl et al., 2008). When the microalgal sequences *OlLPCAT* and *OtLPCAT* were expressed as *V5*-tagged versions in yeast, however, proteins with the expected sizes were not detectable in Western Blot (Figure 4). This result was surprising, as in previous studies expression of *V5-OlLPCAT* fusion constructs in the same yeast strain led to sound protein amounts (Wagner, 2008). Expression conditions, protocol and buffers for homogenate preparation as well as amounts of loaded protein were the same as described before (Wagner, 2008), solely a different proteinase inhibitor was used. One possible explanation is that the expressed protein was not stable in yeast. But as in the positive control of the experiment high levels of complete LacZ-V5p and no significant levels of degradation products were detectable (Figure 4), complete protein degradation for OlLPCAT and OtLPCAT fusion proteins during homogenate preparation can be ruled out. Another possibility to explain the failure of expression tests, at least for *OtLPCAT*, is that protein synthesis terminated co-translationally due to codon usage incompatibilities. Because only the first 14 codons of the sequence were adapted to expression in yeast, inefficient translation of the *OtLPCAT* mRNA might be a reason for missing protein. In order to rule out that the carboxyl-terminal tag led to protein degradation, also lysates from cells expressing amino-terminal *GFP*-tagged versions of *OlLPCAT* and *OtLPCAT* were assayed by Western Blot and by microscopy. These experiments also did not lead to detectable protein levels after expression of both putative LPCAT nucleotide sequences in yeast.

Non-detectable protein can be caused by sensitivity issues connected to the used detection method, still expression of *OlLPCAT* and *OtLPCAT* might have led to functional protein in yeast. Therefore, different LPLAT and thioesterase assays were performed with lysates from expression cultures to investigate the enzymatic activity of OlLPCATp and OtLPCATp. The experimental approach to solving the problem of low expression was the usage of untagged nucleotide sequences which might lead to higher protein levels in yeast. Unfortunately, neither thioesterase nor LPLAT activity was detectable *in vitro* in extracts prepared from cells expressing non-tagged variants of either enzyme, although various combinations of possible acyl donors and acceptors were tested (Table 1). The positive controls were functional, as can be seen from Figure 5, thus the assay conditions were adequate at least for the yeast LPCAT ALE1p. The latter enzyme has been found to mediate broad LPLAT activity *in vitro* (Benghezal et al., 2007; Chen et al., 2007; Jain et al., 2007; Riekhof et al., 2007; Tamaki et al., 2007). This was observed also in the presented experiments (Figure 5; Table 1). For *in vitro* activity of microalgal putative LPCAT proteins, if they were present in the lysates at all, buffer conditions or reaction temperatures might not have been optimal. Maybe also the homogenate preparation procedure influenced their enzymatic activity in a negative manner. Because protein presence in the yeast lysates could not be verified, further optimization attempts of the protocol were not conducted. Due to the described problems, no predictions concerning the effects of expression *in planta* could be made.

4.1.2 OtDGAT2Bp prefers 18:1(n-9)-CoA as substrate *in vitro*

It has previously been shown that expression of *OtDGAT2B* complemented a TAG-deficient yeast mutant strain and the encoded protein *in vivo* accepted polyunsaturated 18- and 20-carbon chain substrates of both the (n-3)- and the (n-6)-series (Wagner, 2008). To verify this rather broad substrate specificity of the microalgal enzyme, the ability of cell homogenates expressing *OtDGAT2B*, *DGA1* or empty vector for TAG formation was tested with various radiolabeled putative acyl donors and two different acyl acceptors. These experiments revealed that *in vitro* di-18:1(n-9)-DAG as well as di-18:2(n-6)-DAG were accepted by OtDGAT2Bp as acyl acceptors when [1-^{14}C]-18:1(n-9)-CoA was used as acyl donor (Figure 6). In contrast to the results obtained from the *in vivo* experiments, however, polyunsaturated acyl-CoAs were not used by OtDGAT2Bp, but only by the yeast enzyme DGA1p. For the latter enzyme, also the observed TAG formation activity *in vitro* was much stronger compared to the microalgal DGAT protein. This could be caused by higher protein amounts upon expression in yeast which was detected before (Wagner, 2008). Furthermore, DGA1p accepted all tested acyl-CoAs with similar preference which reflects also the *in vivo* obtained results for the protein. As 18:1(n-9)-CoA is an endogenous yeast acyl-CoA, it was not possible to compare the incorporation rate into the TAG fraction of yeast cells expressing *OtDGAT2B* for 18:1(n-9)-CoA, as it has been done for the polyunsaturated acyl-CoAs with 18 or 20 carbon atoms (Wagner, 2008). Thus, 18:1(n-9)-CoA possibly could also be the preferred substrate for TAG formation by OtDGAT2Bp *in vivo*. Maybe, *in vitro* reaction conditions were not optimal for OtDGAT2Bp and led to a strongly reduced

DGAT activity compared to the situation *in vivo*. This could explain why no polyunsaturated 18- or 20-carbon chain substrate but only 18:1(n-9)-CoA was accepted by the protein. Shockey and co-workers could show that the tung tree DGAT2 protein also features a distinct substrate preference which strongly influences oil composition of seeds (Shockey et al., 2006). If the OtDGAT2Bp substrate specificity reflected the situation in *O. tauri*, this acyltransferase would not be responsible for the distribution of VLCPUFAs into the microalgal TAG fraction (Wagner et al., 2010). Based on the yeast expression experiments and assuming the catalytic activity of OtDGAT2Bp *in planta* reflects that in yeast, the data obtained so far do not suggest that the enzyme will substantially contribute to VLCPUFA formation in plants.

4.1.3 OtDGAT2Ap features a faint TAG formation ability at low expression temperatures

Previous studies showed that expression of *OtDGAT2A* and *OtDGAT2C* in yeast did not lead to TAG formation in a TAG-deficient yeast mutant. Also, feeding experiments with polyunsaturated fatty acids did not enhance DGAT activity of the encoded proteins (Wagner, 2008). Upon lowering the expression temperatures, however, TAG formation could be promoted in case of OtDGAT2Ap (Figure 7). This could be due to elevated protein stability or activity under temperatures which resemble normal growth conditions of the microalgal gene donor organism. Nevertheless, TAG formation observed upon *OtDGAT2A* expression was not very strong. Following DGAT experiments with homogenates from yeast cells expressing the corresponding sequence did not lead to detectable activity *in vitro* (Figure 9). This might be caused by growing yeast expression cultures at 30 °C or suboptimal assay conditions like too high reaction temperatures or inadequate buffers. Furthermore, DGAT activity of OtDGAT2Ap might have been below the assay detection limit, as it was also barely detectable *in vivo*. This could be also the case for OtDGAT2Cp, whose DGAT activity was neither observable *in vivo* nor *in vitro*. Further experiments investigating possible MGAT or WS activities of OtDGAT2Ap or OtDGAT2Cp led to negative results (Figure 8). This does not rule out possible roles of both enzymes in *O. tauri* because it maybe only reflects the disability of yeast as an expression host which might not provide the necessary reaction environment for the microalgal enzymes. Also, mislocalization or insufficient posttranslational modifications of the proteins can be a problem for proper functioning of the enzymes in yeast. In some studies investigating DGAT2 activities, insect cells were used for expression of the corresponding cDNAs (Lardizabal et al., 2001). These might possibly be more suitable and could be tested also for expression of microalgal DGAT2 sequences. Thus, in *O. tauri* both proteins could still be important for distribution of VLCPUFAs like DHA into the neutral lipid fraction (Wagner et al., 2010). It should be noted that TAG formation in algae is depending strongly on growth stages and environmental conditions (Bigogno et al., 2002; Khozin-Goldberg et al., 2002) which might influence protein activity or stability indirectly. However, because solid enzymatic

activity upon heterologous expression in yeast could not be shown so far, both putative DGAT sequences were not considered for plant expression experiments.

4.1.4 Co-expression of microalgal acyltransferase sequences does not change the enzymatic activity of the encoded proteins

Man and co-workers could show that co-expression of the stearoyl-desaturase SCD1p and DGAT2 protein from mouse in HeLa cells led to enhanced TAG formation *in vivo* due to the interaction of both enzymes (Man et al., 2006a). However, co-expression studies with different acyltransferases in yeast have not been performed up to now. In order to test whether the acyltransferases from *Ostreococcus* display or change their enzymatic activity in yeast upon presence of other acyltransferases from the same organism, it was attempted to co-express *OtLPCAT* or *OlLPCAT* with *OtDGAT2B* or *OtPDAT*. Their effects on acyl-CoA, PC and TAG pool of the yeast expression cultures were investigated subsequently. For the experiments, a yeast mutant lacking endogenous LPCAT, DGAT and PDAT activity was created. This mutant was found to have a different acyl-CoA composition compared to wild type yeast (Figure 10 B). Enhanced levels of 16:1(n-7) and lowered amounts of 18:0 and 18:1(n-9) in the CoA pool of the yeast mutant cultures might permit conclusions concerning the residual yeast enzymatic activities which influence CoA pool composition like LPLATs or acyl-CoA synthetases. Differences in the total amounts of acyl-CoAs, however, were not significant (Figure 10 A). Also the changes in total amounts of PC were not significant and fatty acid composition was the same in the PC fraction of wild type and mutant cells (Figure 12). TAG content was strongly diminished in mutant cells (Figure 11), resulting from the missing DGAT and PDAT activities in the deletion strain.

Co-expression of different combinations of microalgal acyltransferase sequences did not lead to statistically significant differences in the total acyl-CoA amounts and CoA pool composition (Figure 10). Solely, expression of *OtDGAT2B* led to TAG formation in mutant yeast cultures, independently from the co-expressed sequence (Figure 11 A). The fatty acid composition of the neutral lipid fraction, nevertheless, was not changed significantly by the activity of OtDGAT2Bp in comparison to wild type yeast (Figure 11 B). The missing TAG formation upon expression of *OtPDAT* confirmed the results obtained by Dr. Martin Wagner, as also in his experiments TAG formation could not be observed without supplementation of polyunsaturated fatty acids (Wagner, 2008). This enzyme seems to feature a preference for unusual fatty acids, similar to the PDAT1 enzyme from *Arabidopsis* (Stahl et al., 2004).

Especially concerning total amounts and composition of acyl-CoAs and TAG fraction, great differences could be observed between the independent clones expressing the same combination of acyltransferase sequences which led to high standard deviations. Different expression levels might be the reason for the strong variations between the clones. Due to the previously described expression problems concerning putative microalgal LPCAT sequences, it also cannot be ruled out that OlLPCATp or OtLPCATp were not present at all in the yeast expression cultures. Therefore, it cannot be de-

duced from the presented experiments whether the tested microalgal acyltransferases interact with each other or not in yeast.

4.2 Characterization of the microalgal desaturase Old4p in yeast

By seed-specific co-expression of microalgal nucleotide sequences encoding CoA-dependent desaturases with Δ6- and Δ5-specificity together with the elongase sequence *PSE1* (Zank et al., 2002), for the first time exclusively CoA-dependent EPA production with enzymes originating from the plant kingdom could be established in *Arabidopsis* (Hoffmann et al., 2008). In order to produce DHA in a CoA-dependent way, the aim of this work was to identify a Δ4-desaturase with a preference for acyl-CoA substrates. Until now, for this last part of the (n-3)-pathway, three naturally CoA-dependent, Δ5-elongases from microalgal origin have been characterized (Meyer et al., 2004; Pereira et al., 2004b). Also, seven desaturases with Δ4-specificity were found in microalgae during the past years (Qiu et al., 2001; Meyer et al., 2003; Tonon et al., 2003; Pereira et al., 2004b; Tonon et al., 2005b; Zhou et al., 2007; Liu et al., 2007), but none of them so far has been shown to prefer CoA-bound substrates.

4.2.1 The genome of *O. lucimarinus* contains a gene coding for a front-end desaturase

The prasinophyte *O. lucimarinus* was chosen as gene donor, because it is closely related to *O. tauri* and *M. squamata* which both contain CoA-dependent desaturases (Domergue et al., 2005; Hoffmann et al., 2008). Furthermore, the fatty acid composition of *O. lucimarinus* exhibited moderate amounts of the Δ4-desaturation products DHA and 16:4(n-3) (Figure 13). Although DHA amounts were much lower than in *O. tauri* (Wagner et al., 2010), they were similar to those found in *E. gracilis* (Korn, 1964), a microalgal species from which a desaturase with Δ4-specificity has already been identified a few years ago (Meyer et al., 2003).

The protein sequence of the putative desaturase from *O. lucimarinus* was found to contain three histidine boxes with an H to Q substitution in the last box and an amino-terminally fused cytochrome b_5 domain (Figure 15). These are typical characteristics of front-end desaturases (Shanklin et al., 1994; Sperling and Heinz, 2001; Domergue et al., 2002; Napier et al., 2003).

4.2.2 Old4p has a preference for VLCPUFAs and desaturates (n-3)- as well as (n-6)- substrates with Δ4-specificity

When *Old4* was expressed in yeast, the encoded protein was found to introduce double bonds at the Δ4-position and revealed conversion rates of 1 % for 16:3(n-3), 10 % for 22:5(n-3) and 4 % for 22:4(n-6) as substrates (Figure 17). From these results it was deduced that Old4p prefers VLCPUFAs to shorter-chain substrates and fatty acids of

the (n-3)-series are favored. The preference for VLCPUFAs is not reflected by the fatty acid profile of *O. lucimarinus* which clearly contains more 16:4(n-3) than DHA. The preference for (n-3)-substrates, in contrast, would correlate with the fact that only (n-3)-VLCPUFAs can be found in the total fatty acids of the prasinophyte (Figure 13). To be able to classify the obtained conversion rates, also the previously characterized desaturase sequence *Egd4* (Meyer et al., 2003) was expressed in yeast cultures supplemented with the same substrates as those cultures expressing *Old4*. The observed conversion rates in the yeast strain INVSc1 (15 % for 16:3(n-3), 12 % for 22:5(n-3) and 4 % for 22:4(n-6) (Figure 17)) were higher and differed substantially from those obtained by Meyer and co-workers. They performed expression studies in the yeast strain 334 (Hovland et al., 1989) and determined conversion rates of 21 % for 16:3(n-3), about 30 % for 22:5(n-3) and 29 % for 22:4(n-6) (Meyer et al., 2003). In contrast to the results in the yeast strain INVSc1, no clear difference between desaturation of (n-3)- and (n-6)-substrates was detectable. This example indicates that desaturation rates and substrate preference of a heterologously expressed desaturase sequence do not only depend on the encoded enzyme but also on the expression host. Even though such a situation cannot be conclusively ruled out for the experiments performed here, the data indicate that Old4p might be a suitable candidate enzyme to be further tested in transgenic plants.

4.2.3 Old4p acts in a lipid-dependent manner

Because the desaturation product DHA was predominantly present in the PC fraction of yeast cultures expressing *Old4* or *Egd4* (Figure 18), a lipid-dependent desaturation mechanism may be assumed for both tested desaturases. Acyl-CoA-dependent desaturation, like it has been shown for Otd6p, Msd6p and Msd5p (Domergue et al., 2005; Hoffmann et al., 2008), may have led to an equal distribution of DHA between the lipid classes because the CoA pool constitutes the base for synthesis of all different glycerolipids. For Egd4p, Meyer and co-workers found an accumulation of desaturation pro-ducts at the *sn*-2 position of PC and thus proposed a positional specificity for lipid-bound substrates already in their previous studies (Meyer et al., 2003). For Old4p, nevertheless, a lipid-dependency may be surprising due to the close evolutionary relationship of *O. lucimarinus* to *O. tauri*. Therefore, additional co-expression studies were performed to further investigate the substrate specificity of the desaturase. Endogenous CoA-substrate levels should be enhanced in yeast by co-expression of a nucleotide sequence from *T. pseudonana* which encodes a LACS protein specialized on VLCPUFAs (Tonon et al., 2005a). If Old4p acted in a CoA-dependent manner, additional expression of the LACS sequence possibly would have boosted desaturation rates. However, co-expression of *TpLACS* with *Old4* in INVSc1 resulted in lower desaturation rates compared to expression of *Old4* alone (Figure 19). A possible reason for this could be the accumulation of DPA in the neutral lipid fraction. In their studies, Tonon and co-workers observed that the VLCPUFA content in TAGs of yeast cultures expressing *TpLACS* was enhanced six times compared to control cultures (Tonon et al., 2005a). This in turn would limit DPA levels in PC, the desaturation substrate for

lipid-dependent desaturases (Domergue et al., 2003). Nonetheless, Tonon and co-workers only investigated the TAG fraction, but not the phospholipid fractions for presence of VLCPUFAs. In the present study, the amounts of DPA were tested in total lipids and were not found to be enhanced upon expression of *TpLACS*. However, because expression tests were not performed for *TpLACS*, it is not clear whether and in which amounts the encoded protein was present in yeast.

In order to test whether glycolipids are the actual substrates of Old4p, co-expression of the corresponding nucleotide sequence was performed with a cucumber MGDG synthase nucleotide sequence (Shimojima et al., 1997) which had been successfully used in co-expression studies with chloroplastidic desaturases from *A. thaliana* in yeast before (Heilmann et al., 2004). Heterologous expression of this sequence led to the production of MGDG in yeast (Figure 20 A), but incorporation of externally supplied fatty acid into MGDG was only possible for the substrate 16:3(n-3). Co-expression of *Old4* and Cs*MGDGS*, nevertheless, led to a complete lack of desaturation activity in yeast (Figure 20 B). As 16:3(n-3) is one of the most prominent fatty acids in plastidial glycolipids (Browse and Somerville, 1991), it might be favored as substrate by the MGDG synthase. Upon expression of *CsMGDGS* in yeast, this substrate preference could also be the reason for the preferred transfer of 16:3(n-3) into the newly established glycolipid fraction by the MGDG synthase. As Old4p seems to be a PC-dependent desaturase, the potential Δ4-desaturation substrate in this case would be out of range for the enzyme.

Taken together, co-expression studies suggested that missing substrate in shape of acyl-CoAs or galactolipids were not the limiting factors for desaturation in yeast and supported the assumed substrate preference of Old4p for PC-bound acyl chains. Thus, it could either be that *O. lucimarinus* and *O. tauri* contain desaturases with different preferences, although they are closely related to each other. Another possibility would be the co-existence of lipid- and acyl-CoA-dependent desaturases in one organism which has not been shown yet. This could be explained for example by gene duplication and subsequent mutations changing the substrate specificities of the corresponding enzymes. Alternatively, the endosymbiotic uptake of bacteria possessing desaturases with other substrate specificities than those of microalgal enzymes in the course of evolution and the subsequent gene transfer into the nucleus could explain this phenomenon.

4.2.4 Old4p accumulates to solid levels in yeast cells and localizes to their endoplasmic reticulum

Because desaturation rates in yeast were rather low, protein accumulation upon moderate and strong expression of *GFP-Old4* or *GFP-Egd4* was examined by Western Blot studies. These revealed comparable and solid amounts of both fusion proteins (Figure 21). The addition of an amino-terminal epitope, nevertheless, is known to enhance steady-state amounts of plant desaturases upon expression in yeast (O'Quin et

al., 2009). If this is also the case for microalgal desaturases, low protein amounts could still be a problem for untagged versions of Old4p and Egd4p.

Microscopic localization studies were performed to check whether mislocalization or protein aggregation was responsible for the low desaturase activity of Old4p. The GFP-tagged version of the desaturase did not form visible aggregates in yeast cells and seemed to localize to the ER (Figure 22). This would be also the expected localization of a microsomal front-end desaturase. But although the localization at first sight appears to be correct, this still does not rule out the possibility of missing microdomain structures in the yeast ER that are required for proper desaturase function. Also other cases of microalgal desaturases with low activity have been reported (Hoffmann et al., 2008; Lu et al., 2009b; Iskandarov et al., 2010). For all of these enzymes, inappropriate posttranslational modifications, wrong substrate compartmentalization and missing cofactors could be possible reasons for low activity.

Taking together the preceding results, the identified Δ4-desaturase from *O. lucimarinus* was found to act in a lipid-dependent way on (n-3)- as well as (n-6)-substrates with a preference for VLCPUFAs in yeast. However, due to the poor EPA yields obtained so far in *Arabidopsis*, substrate availability for DHA-producing enzymes in those plants would be rather low. Therefore, the enzyme was not further analyzed *in planta*.

4.3 Characterization of acyltransferases, desaturases and elongases in plants

In previous studies, VLCPUFA production was established in plants but resulted in rather low product yields (Hoffmann et al., 2008). These should be improved by the investigation of additional enzymatic activities required for VLCPUFA distribution between different lipid pools and by testing different combinations of the already known enzymes for primary biosynthesis. Also, different plant species were used for VLCPUFA production to analyze the endogenous effects in the host plant seeds.

4.3.1 OlLPCATp increases levels of polyunsaturated fatty acids in the TAG fraction of *Arabidopsis* seeds

After yeast experiments concerning substrate specificities of different acyltransferases, OtDGAT2Bp, OtPDATp, OlLPCATp and OtLPCATp were implemented into plant seed lipid metabolism to find out whether they are able to change TAG levels or composition. Thus, the corresponding cDNA sequences were expressed individually under the control of the *LeB4* promoter in *Arabidopsis* seeds. Regarding TAG content, no differences could be observed between transgenic seeds expressing acyltransferase sequences and seeds expressing empty vector (Figure 23). *OtDGAT2B* expression did also not change the fatty acid composition of total seed lipids (Figure 24 A). In view of the obtained *in vivo* results from yeast which showed no difference between polyunsaturated 18- and 20-carbon fatty acids as substrate for OtDGAT2Bp (Wagner, 2008), strong

changes in the fatty acid composition of seeds were also not expected *in planta* upon *OtDGAT2B* expression. But concerning TAG content, over-expression studies with other fungal or plant DGAT sequences in several plant species including *Arabidopsis* led to enhanced oil deposition (Jako et al., 2001; Lardizabal et al., 2008; Weselake et al., 2008; Zheng et al., 2008; Taylor et al., 2009). In these studies, seed-specific promoters other than the *LeB4* promoter were used which might be a possible reason why expression of *OtDGAT2B* did not result in a similar effect as it has been observed for the other DGAT sequences.

In previous yeast studies, OtPDATp *in vivo* showed TAG forming activity only upon supplementation with polyunsaturated 18- and 20-carbon chain fatty acids. From these results it was deduced that the enzyme might enhance levels of polyunsaturated fatty acids in the TAG pool also in plant seeds (Wagner, 2008). This assumption could not be confirmed. *OtPDAT* expression mostly led to small, but significant changes in saturated and monounsaturated fatty acid levels below 1 % in comparison to control seeds, only 20:1(n-9) levels were increased about 2.2 % (Figure 24 B). Also, *OtLPCAT* expression led to small but significant changes in fatty acid composition (Figure 25 B). For both *OtPDAT* and *OtLPCAT* expression, more independent T2 plant lines should be examined to find out whether the observed changes are consistent.

A possible reason for the completely missing or at most marginal changes observed upon *OtDGAT2B*, *OtPDAT* and *OtLPCAT* expression could be the fact that they were only expressed as partly codon-optimized versions. This could lead to low protein translation rates and subsequently little observable protein activity. As it is assumed that distinct ER regions are dedicated to TAG formation (Shockey et al., 2006; Cahoon et al., 2007), also mislocalization of the microalgal proteins *in planta* could be the reason for low activity. Furthermore, missing posttranslational modifications, cofactors or interacting enzymes that are required for proper enzymatic function are a possible explanation. Faint effects could also be caused by the resilience of the host plant metabolism. In case of minor metabolic changes it has been shown that the plant is able to restore the initial seed lipid composition by endogenous feedback mechanisms (Capell and Christou, 2004).

Seeds expressing the completely codon-optimized version of *OlLPCAT* exhibited the strongest effects observed in this experimental setup. They revealed increased levels of the polyunsaturated 18 carbon-chain fatty acids 18:2(n-6) and 18:3(n-3) and a concomitant decrease in 18:1(n-9), the precursor fatty acid of 18:2(n-6) and 18:3(n-3). A similar observation could be made for the 20 carbon-chain fatty acids 20:1(n-9) and 20:2(n-6). Polyunsaturated fatty acid levels were increased and monoenoic fatty acid amounts were decreased in comparison to the control seeds (Figure 25 A). LPCAT proteins are believed to mediate the transfer of acyl groups between PC and acyl-CoA pool in both directions. However, Stymne and Stobart showed in safflower cotyledons that the transfer from acyl-CoA pool onto the *sn*-2 position of lyso-PC is preferred to the back reaction (Stymne and Stobart, 1984). If the same holds true for the putative microalgal LPCAT protein, it would possibly enhance the levels of monounsaturated fatty

acids at the *sn*-2 position of PC and thereby increase substrate levels for ER-located desaturases. In turn, these could introduce double bonds into the PC-bound acyl substrates and thus produce polyunsaturated fatty acids. Because the changes in fatty acid compositions were localized in the TAG portion of seeds (Figure 26), however, the produced polyunsaturated fatty acids would need to be transported by other enzymatic activities into the TAG fraction. TAG formation in this case could be accomplished directly via an endogenous *Arabidopsis* PDAT activity which produces TAG by transferring the acyl chains from the *sn*-2 position of PC onto the *sn*-3 position of DAG (Figure 1). Alternatively, CPT or PDCT activities could produce DAG molecules enriched in polyunsaturated fatty acids. These DAG molecules in turn would be used for TAG biosynthesis either by a DGAT or a PDAT activity (Figure 1).

In previous studies, *OlLPCAT* was co-expressed together with *Ptd6* and *PSE1* in yeast and was found to mediate the transfer of acyl groups also between PC and acyl-CoA pool, by definition the backwards reaction (Wagner, 2008). If *in planta* this direction would also be preferred by OlLPCATp, the enzyme could possibly remove polyunsaturated fatty acids from the PC pool in the seed and enhance their levels in the acyl-CoA pool. The CoA-bound desaturation products could then be incorporated into TAG by an endogenous plant DGAT activity (Figure 1). From the obtained results, it cannot be concluded which direction of acyl transfer the putative LPCATp from *O. lucimarinus* prefers, because both could in principle be explained with the current TAG biosynthesis model. Nevertheless, the composition of the PC fraction was not affected significantly by expression of *OlLPCAT* (Figure 26). If the polyunsaturated fatty acids are derived from PC, an inverse effect compared to the TAG fraction composition would be expected, resulting in higher levels of monounsaturated fatty acids and lower levels of polyunsaturated fatty acids in this lipid fraction. This effect could again be explained by the resilience of the plant system which leads to generally higher desaturation rates to compensate for the shortfall of unsaturated fatty acids in the membrane lipids. This pronounced adaptation to unforeseen events can also be observed for example as adaptation to lower temperatures in plants. As lowered temperatures lead to more rigid membranes, membrane lipids are desaturated in response to this stimulus to restore membrane fluidity (Ohlrogge and Browse, 1995). A similar effect could be assumed in case of a foreign enzyme activity changing the lipid composition of membrane lipids. It should also be kept in mind that the observed fatty acid composition of total lipids as well as PC and TAG fraction is the final product of all involved endogenous enzymes and the newly introduced enzymatic activity. It would thus be interesting to compare the fatty acid composition of different lipid pools at different time points of seed maturation to find out, which further effects are caused by the foreign enzyme and how the plant responds to it. During these earlier stages, also acyl-CoA composition could be determined to investigate the effects of heterologous *OlLPCAT* expression also in this lipid pool. In summary, the increase of polyunsaturated fatty acids in the seed lipids appeared promising to also test whether co-expression with VLCPUFA-producing enzymes leads to higher yields than the expression of desaturases and elongases alone.

4.3.2 The *Arabidopsis tag1-1* mutant cannot be rescued by expression of different DGAT nucleotide sequences

The *A. thaliana tag1-1* mutant features strongly reduced TAG content and higher levels of 18:3(n-3) at the expense of 18:1(n-9) and 20:1(n-9). The affected gene has been found to encode the AtDGAT1 protein (Katavic et al., 1995; Routaboul et al., 1999; Zou et al., 1999). The effects concerning overall TAG reduction in comparison to wild type seeds observed by other groups were reproducible when plants were cultivated in the climate chamber (Figure 27 A), but not in the greenhouse (Figure 28). Generally, TAG content in wild type seeds was about 100 µg/mg seeds higher upon cultivation in the climate chamber in comparison to the greenhouse. TAG amounts in mutant seeds, however, were equal under the different growth conditions. The difference between both cultivation methods are the more consistent temperature and illumination conditions in the climate chamber, whereas plants are more exposed to seasonal factors in the greenhouse. In case of wild type seeds, the latter growth conditions obviously led to lower TAG accumulation. In case of mutant seeds which already exhibit lowered TAG levels, cultivation conditions did not seem to further influence this factor. Concerning fatty acid composition of wild type and mutant seeds, in contrast, no difference could be observed between climate chamber and greenhouse cultivation. Mutant seeds in both experimental setups revealed the typical flux from the monounsaturated fatty acids 18:1(n-9) and 20:1(n-9) towards the polyunsaturated fatty acid 18:3(n-3) (Figure 27 B and Figure 29). Hence, the fatty acid composition of seed lipids seems to be more independent from the cultivation conditions than the total lipid content.

Surprisingly, expression of the different DGAT nucleotide sequences from *Arabidopsis* or *O. tauri* under the control of the seed-specific *LeB4* promoter did not rescue the mutant phenotype concerning fatty acid composition (Figure 29). At least for the *AtDGAT1* sequence, this was expected because complementation also has been shown in previous studies using the seed-specific *napin* promoter (Jako et al., 2001). There are several possible reasons why the complementation of the mutant was not successful. First, the utilized *LeB4* promoter could be weaker and active in different phases than it was anticipated. The promoter has been isolated from Field Bean (*Vicia faba*) and was shown to drive expression in both *Arabidopsis* endosperm and embryo (Bäumlein et al., 1991b). Nevertheless, there is no information about promoter activity during different stages of embryo development available in *Arabidopsis*. Similar studies have been published only for linseed. They showed that the *LeB4* promoter is active from 11-40 days after flowering (daf) (Drexler et al., 2003). Whether promoter activity is similar in *Arabidopsis* is not clear. Seed maturation in *Arabidopsis* leads to the accumulation of storage compounds and acquisition of dormancy and desiccation tolerance (Goldberg et al., 1994). It can be divided into three stages: The first stage lasts from 7-10 days after pollination (dap) and is important for embryo growth and starch accumulation. In the second stage (11-16 dap), starch is degraded again and instead storage proteins as well as fatty acids are synthesized and deposited in the seed which leads to an increase in seed dry weight. During the third phase (17-20 dap), storage compound bio-

synthesis stops and seeds become quiescent (Baud et al., 2008). For lipid accumulation, thus the second phase is most important. If the promoter activity was the same in *Arabidopsis* as in linseed, it would match well with this second phase. To test promoter activity in *Arabidopsis*, fluorescent reporter genes could be fused to the *LeB4* promoter and the resulting constructs could be transformed into plants. At different developmental stages, transgenic seeds could then be examined for reporter gene expression. Alternatively, transcription of the acyltransferases under control of the *LeB4* promoter could be checked by investigating the mRNA amounts in seeds at different days after pollination.

An alternative approach to complement the observed mutant phenotype would be *AtDGAT1* expression under control of the endogenous *AtDGAT1* promoter instead of the *LeB4* promoter. This would eventually lead to better timed expression. The endogenous promoter could then also be used for expression of *AtDGAT2* and *OtDGAT2B* and thus would lead to more reliable data than the utilization of the *LeB4* promoter.

Astonishingly, in those experiments using wild type *Arabidopsis* plants described in the previous section, effects could be observed upon expression of microalgal acyltransferase sequences under control of the *LeB4* promoter. Apart from problems with transcription intensity and timing, hence also mRNA stability could be an issue influencing protein amounts *in planta*. Inclusion of one or several introns in a gene construct in plants often leads to increased mRNA and subsequent protein accumulation compared to constructs without introns (reviewed in (Koziel et al., 1996; Simpson and Filipowicz, 1996)). In case of the utilized DGAT nucleotide sequences, expression of the gene versions with introns thus might have led to higher protein levels than without introns.

4.3.3 VLCPUFA amounts in transgenic *Arabidopsis* seeds are not stable throughout consecutive generations

Transgenic *Arabidopsis* T2 and T3 seeds containing different combinations of desaturases with the elongase PSE1p were analyzed for their VLCPUFA content. The obtained data showed that independent plant lines representing independent transformation events did not react in the same way. Half of the analyzed plant lines showed higher VLCPUFA amounts in the next generation, whereas the other half showed lower levels. Standard deviations were also not declining in the following generation (Table 3). In the first selection step, those plant lines with the highest amounts of (n-3)-PUFAs were chosen for analysis in the next generation. Most probably, these plant lines did not hold single but multiple insertions. As every insertion segregates separately this leads to strongly varying product yields in the analyzed seeds. Another reason for the differing VLCPUFA amounts could be positional insertion effects. Because insertion of the transformed constructs takes place randomly, fragments can also insert at positions in the genome that are silenced or enhanced in their expression (Kinney et al., 2004). Generally, more independent plant lines with single insertions would need to be tested in order to identify genetically stable plant lines producing solid VLCPUFA amounts throughout consecutive generations.

4.3.4 Combination of Msd6p with lipid-dependent desaturases, PSE1p and OlLPCATp increases EPA levels in transgenic *A. thaliana* seeds

Abbadi and co-workers found out that the effects of co-expression of the lipid-dependent desaturase sequences *Ptd6* and *Ptd5* with the CoA-dependent elongase sequence *PSE1* in plants are limited by a metabolic bottleneck defined by the transfer of the first pathway intermediate between PC- and acyl-CoA pool (Abbadi et al., 2004). This bottleneck was circumvented in subsequent studies by utilization of the acyl-CoA-dependent desaturases Msd6p and Msd5p (Hoffmann et al., 2008). Because of the weak activity of the involved Δ5-desaturase, nevertheless, EPA yields were quite low in *Arabidopsis*. Therefore, the lipid-dependent desaturases and the elongase were combined with the acyl-CoA-dependent desaturase Msd6p to test whether the bottleneck identified before was still present or could be shifted to the next step in the metabolic pathway. In a second approach, *Ptd6*, *Ptd5* and *PSE1* were co-expressed with *OlLPCAT* to investigate the effect of the putative LPCAT enzyme onto transfer of substrates between PC and CoA pool and the resulting VLCPUFA yields. Unfortunately, upon expression in seeds both constructs did not result in higher amounts of product compared to the Pt3 construct alone (data not shown). In a third experiment, all enzymatic activities were tested in combination in seeds. This approach resulted in much higher product yields compared to the Pt3 and the Ms3 constructs tested before (Figure 30). The first intermediate of the pathway, 18:4(n-3), was accumulating at much higher amounts than observed before. This might be caused by the additive effect of the two utilized Δ6-desaturases. The Δ6-elongation product was present at levels comparable to those from seeds expressing Ms3 which reflects the improved substrate flux between Msd6p and PSE1p. 20:5(n-3) levels, after all, were six fold enhanced in seeds expressing the OlLPCAT-Msd6-Pt3 construct compared to seeds expressing Pt3 or Ms3. This effect could be caused by the higher desaturation activity of Ptd5p compared to Msd5p. In this case, the transfer from CoA pool into PC pool would not be the rate-limiting step in the VLCPUFA biosynthesis pathway. An additional factor potentially influencing product yields is the activity of OlLPCATp. This enzyme might enhance transfer rates between different lipid pools and thereby positively influence VLCPUFA yields. However, when both Msd6p and OlLPCATp were combined alone with the lipid-dependent desaturases and the elongase, none of these effects could be observed. Therefore, the number of tested transgenic lines was either too low for Msd6-Pt3 and OlLPCAT-Pt3 or the effects are only achieved when both sequences are combined with the Pt3 construct. The presented values of Pt3 and Ms3 seeds are taken from (Hoffmann et al., 2008). To be able to systematically compare VLCPUFA yields in the diffe-rent transgenic seeds, several independent plant lines expressing Pt3, Ms3 or the respective combination constructs would need to be cultivated in parallel to have the same environmental factors like illumination and temperatures which might also influence product amounts.

4.3.5 *Camelina* is a promising oilseed crop for VLCPUFA production

The oilseed crop *C. sativa* has not been cultivated extensively for some decades, but recently has experienced a comeback (Lu and Kang, 2008). The reason for this fact is the elevated 18:3(n-3) content in *Camelina* seeds and the resulting health-promoting high (n-3) over (n-6) ratio (Figure 31). Due to its outstanding 18:3(n-3) levels, the plant species was considered as good choice especially for the production of (n-3)-VLCPUFAs and therefore transformed with the VLCPUFA-construct Pt3. Alongside, *Arabidopsis* seeds expressing the Pt3 construct were analyzed. Results obtained for *A. thaliana* seeds were, especially in the case of T3 seeds, much lower than in the EPA screen described in section 3.3.3 (Table 3, Figure 32 and Figure 33). For both experiments, different plant lines were used. This once again shows the large variation between independent transformation events. Furthermore, the EPA screen results were obtained by pool analysis, whereas in the here described experiments single seeds were analyzed. Analysis of seed pools always gives mean values of a mixture from seeds featuring different numbers of insertions. Every result after single seed analysis, in contrast, is derived just from one seed. Thus, this could also explain the different results obtained by different analysis methods.

Generally, VLCPUFA percentages from total fatty acids were higher in *Arabidopsis* T2 seeds compared to *Camelina* T2 seeds (Figure 32). This, however, changed in the subsequent generation, in which *Camelina* seeds contained higher VLCPUFAs percentages than *Arabidopsis* seeds in their total fatty acids (Figure 33). This could be due to genetic effects which maybe lead to a more stable integration of constructs into the *Camelina* genome than into the genome of *Arabidopsis*. However, one should consider that T3 *Camelina* seeds looked shriveled and had a much lower content of the most prominent fatty acid 18:3(n-3) in total fatty acids. Also, the initial precursor fatty acid for both (n-3)- and (n-6)-VLCPUFAs, 18:1(n-9), in total fatty acids was lower compared to wild type seeds (Figure 31 and Figure 33). Because the percentages of VLCPUFAs were calculated on the basis of total fatty acids, absolute product amounts in both *Arabidopsis* and *Camelina* seeds would need to be determined by utilization of internal standard to be able to compare them.

Although *C. sativa* seed lipids have a higher (n-3) over (n-6) ratio than *A. thaliana* seed lipids (Figure 31), much more (n-6)-VLCPUFAs than (n-3)-VLCPUFAs were produced in *Camelina* seeds (Figure 32 and Figure 33). This observation could be explained by differences in substrate availability. The lipid-dependent desaturase Ptd6p requires PC-bound substrates for its enzymatic activity (Domergue et al., 2003). If more 18:2(n-6) than 18:3(n-3) is available in the PC pool, this in turn would lead to higher product amounts of the (n-6)-series. 18:3(n-3), on the other hand, might be transferred directly after its synthesis into the TAG fraction and thus would not be available for further enzymatic activities.

From the presented results it can be deduced that in principle VLCPUFA production in the oilseed crop *C. sativa* is possible and leads to reasonable product yields. Nevertheless, transformation efficiency for this plant species was quite low. That is why only a

few independent plant lines could be analyzed. More lines and constructs with different promoters and genes would need to be tested in consecutive generations to find out which combinations are most effective.

4.3.6 Combination of microalgal acyltransferases and primary VLCPUFA biosynthesis activities does not lead to higher product yields

After the independent analysis of acyltransferases and VLCPUFA-producing enzymes *in planta*, combinations of both were tested in *Arabidopsis* wild type as well as *tag1-1* seeds. Due to the transformation of two different constructs which inserted separately into the plant genome, two sequential selection steps were required. This only led to a small number of surviving plants for subsequent analysis. Large variations in VLCPUFA yields were observable for independent plant lines and might be caused by multiple insertions or positional effects of Pt3 insertion. TAG contents were not affected by co-expression of acyltransferase sequences with Pt3 in both wild type and mutant seeds (Figure 34 and Figure 37). Resulting VLCPUFA yields were also not different in seeds co-expressing acyltransferases compared to those expressing empty vector with the Pt3 construct. This can have several reasons. First, the issues discussed for acyltransferases in the previous sections hold true in the same way. Furthermore, in both constructs, different promoters were used. Whereas the *LeB4* promoter was driving expression of acyltransferase sequences, the *USP* promoter was used to drive expression of *Ptd6*, *PSE1* and *Ptd5*. The latter one has also been isolated from *V. faba* and was shown to be expressed in the *Arabidopsis* embryo (Bäumlein et al., 1991a). In linseed, the *USP* promoter was found to be active during 5-40 daf, six days earlier than the *LeB4* promoter (Drexler et al., 2003). If the same holds true for expression in *Arabidopsis*, both promoters should have a long-lasting overlapping activity and thus should be suitable for combined utilization. If in *Arabidopsis*, however, expression activity of both promoters would be different than in linseed, this could be a possible reason for the missing effect of acyltransferases onto VLCPUFA yields.

In previous studies it was shown that the desaturase SCD1p and the acyltransferase DGAT2p *in vivo* interact with each other (Man et al., 2006a). The enzymes from *O. tauri* and *O. lucimarinus*, in contrast, most probably do not interact with the desaturases from *P. tricornutum*. This could be the reason for the absent effect of co-expression. Utilization of enzymatic activities from one gene donor, on the other hand, might possibly have led to an observable increase in VLCPUFA yields.

In case of the *tag1-1* mutant seeds which feature higher 18:3(n-3) levels than wild type seeds (Figure 27 B), higher amounts of (n-3)-VLCPUFAs were expected than in the wild type. These were, similarly to the situation in *Camelina* seeds, not observable. The reasons for this result could be the same as discussed above for *Camelina*. Maybe, substrate availability, but not total amounts of the relevant fatty acid are rate-limiting for further modifications.

4.4 Future perspectives

Since the first attempts about 15 years ago, fundamental progress has been made towards the aim of VLCPUFA production in plants. At the same time, also a deeper insight into seed lipid metabolism has evolved. Nevertheless, not all processes occurring during seed maturation and synthesis as well as distribution of unusual fatty acids are as yet fully understood. As an example, recently a completely new enzymatic activity was identified to play a key role in seed lipid metabolism by transferring the headgroup of PC onto DAG and *vice versa* (Figure 1) (Lu et al., 2009a). As long as the seed lipid metabolism is not completely known, it is of course difficult to find the right switch to change it according to the corresponding needs. Absolute yields might be increased by a metabolic pull function which distributes products into storage lipids, where they are not as strongly accessible by further enzymatic activities than in other pools (Cahoon et al., 2007). This metabolic pull function needs to be highly specific to not transfer already intermediates of the production pathway, but only the end product. DGATs or PDATs with a preference for EPA or DHA for example would be helpful tools to improve the metabolic flux of substrates through the VLCPUFA pathway. These enzymes might be identified by investigating lipid composition and fatty acid distribution into different lipid fractions from potential candidate organisms. The expression pattern of the genes of interest should be investigated in-depth, especially in phases, in which changes in the fatty acid composition or distribution become visible. This may allow conclusions concerning the substrate specificity of the enzymes in question.

As the acyl-CoA pool plays a central role in lipid metabolism, it is also of vital importance for the production of unusual fatty acids. In plants, this lipid pool is assumed to be rather low compared to yeast or animals (Larson and Graham, 2001). Therefore, enhancing the flux of fatty acids into this pool might be a useful means to enhance product yields (Venegas-Calerón et al., 2010). However, because also in this respect the current understanding is rather limited and the current model of direct export of products from the plastidial FAS complex into the cytosolic CoA pool has been questioned recently (Bates et al., 2007), further research is required also in this field of lipid metabolism (Venegas-Calerón et al., 2010).

The transfer of substrates between acyl-CoA pool and PC pool represents a bottleneck in VLCPUFA-production in plants (Abbadi et al., 2004). A solution for this problem would be the identification of a LPCAT enzyme specific for the substrates of VLCPUFA-biosynthesis. Alternatively, highly active acyl-CoA-dependent desaturases with Δ5- and Δ4-specificity would need to be identified to circumvent the described bottleneck.

Clearly, VLCPUFA-biosynthesis in plants has to be accomplished by a concerted expression of several primary and secondary enzymatic activities that need to cooperate in the host organism. This requires utilization of balanced promoters which are active during the phase of seed maturation to assure optimal product yields. To find out, which promoters are suited best for this goal, large-scale promoter studies need to be

performed. These need to be done for every potential VLCPUFA-producing host species independently, because promoter activity might vary between different organisms. As it is known that using the same promoter several times in one construct can lead to silencing effects, adequate combinations of different promoters have to be identified. Currently, the promoters used for seed-specific expression of genes are derived from storage protein synthesis. It might also be worth to isolate promoters involved in lipid synthesis and oil accumulation during seed development (Venegas-Calerón et al., 2010).

Upon expression of foreign genes in a heterologous system, compartmentalization of substrates and enzymes turned out to be problematic (Graham et al., 2007). It was also proposed that there are several CoA pools in the cell which are not accessible by all lipid-modifying enzymes (Dyer and Mullen, 2008). These issues further complicate the production of a desired fatty acid product in huge amounts. For optimal product yields, introduced enzymatic activities need to be co-localized to have an optimal metabolic flux through the biosynthesis pathway, as it has for example been realized by nature for the FAS complex or the PKS-like system. Investigating these complex structures might lead to transferable solutions to overcome the presented problems.

5 Summary and Conclusions

The production of dietary important VLCPUFAs like EPA or DHA in annual oilseed crops represents a valuable substitute for fish stocks as declining natural sources of these fatty acids. However, there are still hurdles which need to be overcome, especially concerning absolute VLCPUFA yields and distribution of the end products into the neutral lipid fraction of seeds. The implementation of specific acyltransferases and highly active desaturases from primary producers of VLCPUFAs might help to optimize these processes.

Several nucleotide sequences encoding potential acyltransferases from *O. lucimarinus* and *O. tauri* were characterized by *in vivo* and *in vitro* experiments in yeast. Two microalgal DGAT enzymes, OtDGAT2Ap and OtDGAT2Bp, were found to complement a yeast mutant deficient in TAG synthesis. OtDGAT2Ap was only active at expression temperatures between 16 °C and 23 °C, whereas OtDGAT2Bp produced TAG also upon expression at 30 °C. The latter was the only DGAT enzyme revealing activity *in vitro*. It preferred monounsaturated 18 carbon-chain fatty acids as substrate and did not show any activity towards VLCPUFAs.

In addition, the nucleotide sequence *Old4* encoding a front-end desaturase with Δ4-specificity was isolated from the microalgal species *O. lucimarinus* and characterized by heterologous expression in yeast. The enzyme Old4p was found to desaturate substrates of the (n-3)- and the (n-6)-series in a lipid-dependent way and preferred VLCPUFAs to shorter-chain fatty acids.

Those acyltransferases with the strongest activity, demonstrated in preceding yeast experiments, were introduced individually and in combination with VLCPUFA-producing enzymes into *A. thaliana* to investigate their interplay with seed metabolism. Upon expression of the microalgal nucleotide sequence *OlLPCAT* from *O. lucimarinus*, absolute amounts of polyunsaturated fatty acids were elevated in the TAG fraction of seeds. In contrast, expression of all other tested acyltransferase sequences did not result in a change of fatty acid composition in seeds. Further co-expression studies with sequences encoding enzymes necessary for VLCPUFA-biosynthesis did not show a positive effect of acyltransferases on VLCPUFA yields. Besides the analysis of microalgal acyltransferases *in planta*, with *A. thaliana* and *C. sativa* also two different host plant species were investigated for VLCPUFA-production to compare the effects of endogenous acyltransferases onto product yields. In these studies, *Camelina* was identified as a promising oilseed crop for heterologous biosynthesis of VLCPUFAs.

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Appendix

The following tables give the sequences of primers used for isolation and cloning of genes. Primer sequences are given in the 5' to 3' orientation and restriction sites for cloning into expression plasmids are in bold.

Appendix 1. *OlLPCAT* and *OtLPCAT*

Name	Sequence
OlLPCATcoforBamHI	ATGC**GGATCC**ACATAATGTTATCTGCCGCCGCATTTGG
OlLPCATcorevApaI	ATGC**GGGCCC**TTAGGCAGACTTAGGTTTACTTGACC
OlLPCATcorevNotI	GCAT**GCGGCCGC**TTAGGCAGACTTAGGTTTACTTGACC
OlLPCATcoforHindIII	CCGGATGC**AAGCTT**ATGTTATCTGCCGCCGCATTTGG
OlLPCATcorevXhoI	CCGGATGC**CTCGAG**TTAGGCAGACTTAGGTTTACTTGACC
OtLPCATtcoforBamHI	CCGGATGC**GGATCC**ATGGACGCTGCTTTGGACGC
OtLPCATrevNheI	CCGGATGC**GCTAGC**TTAAGCCGTCTTGGGTTTGCTCG
OtLPCATtcorevNotI	CCGGATGC**GCGGCCGC**TTAAGCCGTCTTGGGTTTGCTCG
OtLPCATtcorev-StopXbaI	CCGGATGC**TCTAGA**AGCCGTCTTGGGTTTGCTCG
OtLPCATtcoforSpeI	CCGGATGC**ACTAGT**ATGGACGCTGCTTTGGACGC
OtLPCATtcorevHindIII	CCGGATGC**AAGCTT**TTAAGCCGTCTTGGGTTTGCTCG

Appendix 2. *OtDGAT2A*, *OtDGAT2B* and *OtDGAT2C*

Name	Sequence
OtDGAT2AcoforEcoRI	ATGC**GAATTC**ACATAATGTCCAGACCAGAACCAGGAAGTC
OtDGAT2AcorevNotI	GCAT**GCGGCCGC**TTATACCAATTTAGTTCTCTGAGTTGG
OtDGAT2BtcoforBamHI	ATGC**GGATCC**ACATAATGGGTTCCAGATCTATTGTTGACC
OtDGAT2BtcorevNotI	GCAT**GCGGCCGC**TCAGCACACGACGAGGTCGACG
OtDGAT2CcoforEcoRI	ATGC**GAATTC**ACATAATGATTTATGCATTTTTGTTGTCAGC
OtDGAT2CcorevNotI	GCAT**GCGGCCGC**CTACTTAAATGTCATAGCTTGC

Appendix 3. *OtPDAT*

Name	Sequence
OtPDATtcoforEcoRI	ATGC**GAATTC**ACATAATGGCTGTTACTAGAAGAACTACTAG
OtPDATtcorevNotI	GCAT**GCGGCCGC**CTAAAACGACTCGCGTGGGTTGG

Appendix 4. *Old4*

Name	Sequence
Old4for	ATGCCCTCCGCCGCGCGCTCCCGAG
Old4hpggfor	CATCGCCATCCCGGCGGCGCGCAATTG
Old4mfor	GAGTGGCGGTATCATCACATGGTGAG
Old4hpggrev	CAATTGCGCGCCGCCGGGATGGCGATG
Old4mrev	CTCACCATGTGATGATACCGCCACTC
Old4rev	CTACATGGCCTTCGGTCGTTGATGG
Old4revNotI	GCAT**GCGGCCGC**CTACATGGCCTTCGGTCGTTGATGG
Old4tcofor	ATGCCATCTGCTGCTAGATCTAGAGCTTCTAAAAGAGCTAA TGCTACTACTGATGTTGCTACGACCGCTCCCGAGG-CGACGCTCG
Old4tcoforEcoRI	CCGGATGC**GAATTC**ATGCCATCTGCTGCTAGATCTAG
Old4revXhoI	CCGGATGC**CTCGAG**CTACATGGCCTTCGGTCGTTGATG

Appendix 5. *Egd4*

Name	Sequence
Egd4forBamHI	A**GGATCC**AGATCTCACCATGTTGGTGCTGTTTGGCAATT-TCTATG
Egd4revXhoI	A**CTCGAG**CCCGGGTTATGACTTTTTGTCCCCGTTCGTTG
Egd4forEcoRI	CCGGATGC**GAATTC**ATGTTGGTGCTGTTTGGCAATTTC

Appendix 6. *TpLACS*

Name	Sequence
TpLACSfor	ATGACGAACAAATGGTCCATAGAAG
TpLACSrev	TTACAACTTGCTCTGAGGAGAATC
TpLACSforApaI	ATGC**GGGCCC**ACATAATGACGAACAAATGGTCCATAGAAG
TpLACSrevNheI	GCAT**GCTAGC**TTACAACTTGCTCTGAGGAGAATC

Appendix 7. *CsMGDGS*

Name	Sequence
CsMGDGSforBamHI	CCGGATGC**GGATCC**ACATAATGGGTGTTTCAGATGAAAC-CAATGG
CsMGDGSrevNheI	CCGGATGC**GCTAGC**TCAGCCGGAATATTGTGGTACAA-AAC

Appendix 8. *AtDGAT1* and *AtDGAT2*

Name	Sequence
AtDGAT1forKpnI	CCGGATGC**GGTACC**ATGGCGATTTTGGATTCTGCTGG
AtDGAT1revXhoI	CCGGATGC**CTCGAG**TCATGACATCGATCCTTTTCGG
AtDGAT2forEcoRI	CCGGATGC**GAATTC**ATGGGTGGTTCCAGAGAGTTCCG
AtDGAT2revXhoI	CCGGATGC**CTCGAG**TCAAAGAATTTTCAGCTCAAG

Curriculum Vitae

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Education and Qualifications

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Presentations at International Conferences

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2009	Poster at the 4th European Symposium on Plant Lipids (Göttingen, Germany) Poster at the 7th Euro Fed Lipid Congress (Graz, Austria)
2008	Oral presentation at the 6th Euro Fed Lipid Congress (Athens, Greece)

Stipends

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10/2008-09/2010	"Chemiefonds-Stipendium" from the "Verband der Chemischen Industrie (VCI)"
10/2007-09/2008	Georg-Christoph-Lichtenberg-Stipend from the state Lower Saxony
10/2006-08/2007	Stipend from the International Max Planck Research School
02/2006-03/2008	Stipend from the German National Academic Foundation