Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2020:24
Faculty of Landscape Architecture, Horticulture and Crop Production Science

The specificities of plant enzymes and their effects on the seed oil quality

Simon Jeppson

The specificities of plant enzymes and their effects on the seed oil quality

Simon Jeppson
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding
Alnarp

Doctoral thesis
Swedish University of Agricultural Sciences
Alnarp 2020

Acta Universitatis Agriculturae Sueciae
2020:24

“The madder something is, the better it works," the Cheshire cat said. "Which is of course, why you fit in very well.” - Cover photo: Simon Jeppson

ISSN 1652-6880
ISBN (print version) 978-91-7760-566-9
ISBN (electronic version) 978-91-7760-567-6

Print: SLU Service/Repro, Alnarp 2020

The specificities of plant enzymes and their effects on the seed oil quality

Abstract

Triacylglycerols, glycerides with three fatty acids, are the chemical constituent of seed oils. The fatty acid composition determines the chemical and physical characteristics of an oil. Conventional vegetable oils contain a few reoccurring fatty acids, but plants can produce several hundreds of different fatty acids. A wide range of plant oils and their fatty acids with specific characteristics are suitable replacements for many fossil-oil based commodities. Plant breeders and researcher are thus, modifying the fatty acid composition in oil crops to enhance such properties. Erucic acid is a fatty acid, with extensive use within industrial applications. Fatty acids originate from the plastids in plants, but the assembly of triacylglycerols occurs in the endoplasmic reticulum, where integral membrane-bound acyltransferases catalyse the formation of triacylglycerols. Modifications of fatty acids occur while they are associated with specific lipid molecules. Different types of fatty acid modifications require association with different lipid molecules, and several unusual fatty acids require multiple sequential modifications. The intricate system of synthesis, modification and assembly thus requires channelling of fatty acids and substrates from different subcellular compartments and concurrent transfer of fatty acids from various lipid molecules, often at multiple occasions. The enzymes governing these processes often have very distinct substrate specificities, which we investigate here. Re-engineering of a seed oil's fatty acid composition or the introduction of an exotic fatty acid is a complex process and must take into consideration plants endogenous enzymes, their substrate specificities and the available substrate pools. Detailed biochemical characterisations of crucial enzymes are thus essential for a successful outcome of such efforts. We have thoroughly investigated a range of acyltransferases and other enzymes involved in lipid metabolism, mainly focused towards erucic acid in several plant species, including Arabidopsis, *Camelina sativa*, *Brassica napus*, *Crambe hispanica* ssp. *abyssinica* and *Tropaeolum majus*. DGAT2 catalyses the acyl-CoA dependent formation of triacylglycerol and is thus one of the acyltransferases involved in oil synthesis. The assessed substrate specificities of several DGAT2 indicate that its substrate specificity is a bottleneck in the further increase of erucic acid. We have also investigated the amino acid sequence motifs governing the specificity towards erucic acid in DGAT2 enzymes.

Keywords: Plant lipids, TAG, DGAT, acyltransferase, lipid synthesis, enzyme specificities, erucic acid, oil crop

Author's address: Simon Jeppson, SLU, Department of Plant Breeding,
P.O. Box 101, 230 53 Alnarp, Sweden

Växtenzyms specificiteter och deras effekter på fröoljekvaliteten

Abstrakt

Triglycerider, en glycerid med tre fettsyror, är den kemiska beståndsdelen i fröoljor. Fettsyrasammansättningen avgör oljors kemiska och fysikaliska egenskaper. Konventionella vegetabiliska oljor innehåller några få återkommande fettsyror, men växter kan producera flera hundratals olika fettsyror. Många växtoljor och deras fettsyror med specifika egenskaper är lämpliga som ersättning till många fossila oljebaserade råvaror. Växtförädlare och forskare modifierar därför fettsyrasammansättningen i oljeväxter för att förbättra deras industriella egenskaper. Erukasyra är en fettsyra, med omfattande användning inom industriella tillämpningar. Fettsyror härstammar från plastiderna i växter, men sammansättningen av triglycerider sker i endoplasmatisk retikulum, där membran-integrerade acyltransferaser katalyserar syntesen av olja. Modifieringar av fettsyror sker medan de är associerade med specifika lipidmolekyler. Olika typer av fettsyramodifikationer kräver associering med olika molekyler, och flera ovanliga fettsyror kräver flera sekventiella modifieringar. Det komplicerade systemet för syntes, modifiering och montering kräver därför kanalisering av fettsyror och substrat från olika subcellulära organ och samtidigt en överföring av fettsyror från olika lipidmolekyler, ofta vid flera tillfällen. Enzymerna som styr dessa processer har ofta mycket distinkta substratspecificiteter som vi undersökt här. Förändring av fröoljans fettsyrasammansättning eller introduktion av en exotisk fettsyra är en komplex process där man måste ta hänsyn till växternas endogena enzymer, deras substratspecificitet och de tillgängliga substratpoolerna. Detaljerade biokemiska karakteriseringar av viktiga enzymer är således avgörande för ett framgångsrikt resultat. Vi har grundligt undersökt ett antal acyltransferaser och andra enzymer involverade i lipidmetabolismen, huvudsakligen inriktad på erukasyra i flera växtarter, inklusive Arabidopsis, *Camelina sativa*, *Brassica napus*, *Crambe hispanica* ssp. *abyssinica* och *Tropaeolum majus*. DGAT2 katalyserar den acyl-CoA-beroende bildningen av triglycerider och är således ett av de viktiga acyltransferaserna involverade i syntesen av olja. Undersökningana indikerar att flera DGAT2 enzymers substratspecificitet utgör ett hinder för ytterligare ökning av erukasyrahalten i fröoljan. Vi har också undersökt aminosyrasekvensmotiv som styr specificiteten gentemot erukasyra i DGAT2.

Nyckelord: Växtlipider, TAG, DGAT, acyltransferase, lipidsyntes, enzym specificitet, erukasyra, oljegröda

Författarens adress: Simon Jeppson, SLU, Institutionen för växtförädling,
Box 101, 230 53 Alnarp, Sverige

Dedication

To all the giants upon whose shoulders I stand

Рукописи не горят - Manuscripts do not burn
Mikhail Afanasyevich Bulgakov

Contents

List of publications **9**

List of figures **11**

Abbreviations **13**

1 Preamble **15**

2 Introduction **17**
2.1 Fatty acid synthesis in plants 21
2.2 Export of acyl-chains from the plastid 24
2.3 TAG assembly in the endoplasmic reticulum 26
2.4 Acyl import to the plastids 30
2.5 Unusual fatty acid modifications 33
2.6 Engineered seed oil quality through conventional plant breeding. 39
2.7 Engineered seed oil quality through a biotechnological approach 40

3 Principles and justification of reoccurring methods **47**

4 Results and insights derived from the papers **51**

5 Future perspectives and discussion **63**

References **67**

Popular science summary **75**

Populärvetenskaplig sammanfattning **77**

Acknowledgements **79**

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Demski, Kamil, Jeppson, Simon, Lager, Ida, Misztak, Agnieszka, Jasieniecka-Gazarkiewicz, Katarzyna, Waleron, Małgorzata, Stymne, Sten, Banaś, Antoni. "Isoforms of acyl-CoA: diacylglycerol acyltransferase2 differ substantially in their specificities toward erucic acid." Plant Physiology 181, no. 4 (2019): 1468-1479.

II Jeppson, Simon, Mattisson, Helena, Demski, Kamil, Lager, Ida. ”A predicted transmembrane region in plant diacylglycerol acyltransferase 2 regulates the specificity towards very long chain acyl-CoAs.”, Submitted manuscript.

III Jeppson, Simon, Kamil Demski, Anders S. Carlsson, Li-Hua Zhu, Antoni Banaś, Sten Stymne, and Ida Lager. "Crambe hispanica subsp. abyssinica diacylglycerol acyltransferase specificities towards diacylglycerols and acyl-CoA reveal combinatorial effects that greatly affect enzymatic activity and specificity." Frontiers in Plant Science 10 (2019): 1442.

IV Jeppson, Simon, Rivani, Meta, Lager, Ida. ” Identification of DGAT2 and characterisation of DGAT enzymes from Tropaeolum majus.”, Manuscript.

V Lager, Ida, Jeppson, Simon, Stymne, Sten, Marmon, Sofia. ”Acyltransferases regulate oil quality in Camelina through both acyl donor and acyl acceptor specificities.”, Submitted manuscript.

The contribution of S.J to the papers included in this thesis was as follows:

I K.D., **S.J**., I.L., S.S., and A.B. conceived the research plans; K.D., **S.J**., I.L., and A.B. designed the experiments; I.L., S.S., and A.B. supervised the experiments; A.M. and M.W. designed and performed the phylogenetic analyses; K.D., **S.J**., I.L., and K.J.-G. performed the experiments; K.D., S.J., and I.L. analyzed the obtained data; K.D., **S.J**., A.B., I.L., and S.S. wrote the article; K.D & **S.J** contributed equally

II I.L., **S.J.**, and K.D. perceived the concept; I.L. and **S.J.** designed the experiment; I.L., H.M., and **S.J.** conducted the experiments; **S.J.** wrote the original draft; I.L., **S.J.**, H.M., and K.D. reviewed and edited the final version.

III I.L. and S.S. envisioned this project; **S.J.**, I.L., S.S., and K.D. coordinated and designed the experiments; **S.J.**, K.D., and I.L. drafted the manuscript; A.B., A.C., L.-H.Z., and S.S. contributed to the final version; K.D. & **S.J.** contributed equally.

IV I.L. and **S.J.** perceived the concept; I.L. and **S.J.** designed the experiments; I.L., **S.J**., and M.R. conducted the experiments; **S.J.** and I.L. wrote the manuscript; I.L., **S.J.**, and M.R. reviewed and edited the final version.

V S.M, **S.J.**, I.L., I.F. and S.S. conceived the original research plan; I.L., **S.J.**, A-L.G., S.M., and S.S. performed the experiments; S.S. wrote the article with contributions from all the authors.

List of figures

Figure 1 The chemical structures for TAG, oleic acid and wax ester. **18**

Figure 2 Schematic overview of the cyclic reactions catalysing acyl elongation in FAS.....**22**

Figure 3 Overview of the reactions catalysed by the KAS enzymes during the condensation in FAS.....**23**

Figure 4 Overview of the synthesis of FAs in the plastid and the following export to the cytosol through proposed mechanisms.....**25**

Figure 5 Simplified overview of TAG assembly in the ER.....**27**

Figure 6 Overview of two alternative metabolic routes present in Arabidopsis for the synthesis of thylakoid membrane lipids.....**32**

Figure 7 Structural features of several UFAs.....**35**

Figure 8 The proposed biosynthetic pathway, resulting in nebraskanic acid in *O. violaceus*. **38**

Figure 9 Metabolic routes in the aerobic pathway for the synthesis of both EPA and DHA **44**

Figure 10 Principles behind expressing TAG producing acyltransferases in H1246 .....**48**

Figure 11 Acyl-CoA specificities of two *B. napus* DGAT2 enzymes and phylogenetic analysis of these, including both ancestral parents DGAT2 sequences.....**54**

Figure 12 Parental wildtype enzymes and chimeric DGAT2 enzymes and their specificity towards 18:3$\Delta^{9,12,15}$ and 22:1Δ^{13} **56**

Figure 13 Illustration of the *Bna*A.DGAT2.d amino acid sequence with essential features.....**57**

Figure 14 Substrate specificities of crambe DGAT enzymes towards 22:1Δ^{13}-CoA in combination with the frequently occurring *sn*-1-22:1Δ^{13}-*sn*-2-18:1Δ^{9}-DAG or di-22:1Δ^{13}-DAG.....**58**

Figure 15 Substrate specificities of *T. majus* DGAT enzymes towards 22:1Δ^{13}-CoA in combination with most preferred DAG species or di-22:1Δ^{13}-DAG **59**

Figure 16 Acyl-CoA specificity of CsDGAT2 and a phylogenetic analysis including the *B. napus* DGAT2 amino acid sequences **61**

Abbreviations

ACC	acetyl-CoA carboxylase
ACP	acyl carrier protein
ADS	acyl-CoA desaturase-like
ANOVA	analysis of variance
BASF	badische anilin- und soda-fabrik
Cas9	CRISPR-associated protein 9
CPT	CDP-choline:DAG cholinephosphotransferase
CRISPR	clustered regularly interspaced short palindromic repeats
CSIRO	commonwealth scientific and industrial research organisation
DAG	diacylglycerol
DGAT	acyl-CoA:diacylglycerol acyltransferase
DGDG	digalactosyl diacylglycerol
DHA	docosahexaenoic acid
DSM	dutch state mines
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FA(s)	fatty acid(s)
FAS	fatty acid synthase
FFA(s)	free fatty acid(s)
GPAT	glycerol-3-phosphate acyltransferase
GPC	glycerol-3-phosphocholine
GPCAT	glycerophosphocholine acyltransferase
GRDC	grains research and development corporation
HEAR	high erucic acid rapeseed
HELP	high erucic low polyunsaturated

HPLC	high-performance liquid chromatography
HSD	honestly significant difference
KAS	β-ketoacyl-ACP synthase
KCS	β-ketoacyl-CoA synthase
LACS	long-chain acyl-CoA synthetases
LC-PUFA	long-chain ω-3
LCS	liquid scintillation counter
LEAR	low erucic acid rapeseed
LLC	limited liability company
LPA	lysophosphatidic acid
LPAT	lysophosphatidic acid acyltransferase
LPC	lysophosphatidylcholine
LPCAT	lysophosphatidylcholine acyltransferase
MCFA	medium-chain fatty acids
MCMT	malonyl-CoA:acyl carrier protein malonyltransferase
MGDG	monogalactosyl diacylglycerol
MSY	maximum sustainable yield
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PDAT	phospholipid: diacylglycerol acyltransferase
PDCT	phosphatidylcholine diacylglycerol cholinephosphotransferase
PE	phosphatidylethanolamine
PGP	phosphatidyglycerophosphate
PI	phosphatidylinositol
PKS	polyketide synthases
PS	phosphatidylserine
PUFA(s)	polyunsaturated FA(s)
RACE	rapid amplification of cDNA ends
SAD	Δ^9 stearoyl-ACP desaturase
SQDG	sulfoquinovosyl diacylglycerol
TAG	triacylglycerol
TLC	thin-layer chromatography
UFA(s)	unusual fatty acid(s)
VLCFA	very long-chain fatty acids

1 Preamble

Many fatty acids (FAs) have trivial names usually associated with either a property of the FA or a common source from where the FA is derived. Examples of trivial names include, e.g. caproic acid as it smells of goat (Capricornus means horned goat in Latin), palmitic acid (often associated with palm oil) and linolenic acid (linon is Greek for flax, which produces an oil containing linolenic acid). The trivial names are tedious to learn and seldom reveal any essential structural properties of the FAs. Most often the Δ nomenclature is used instead since it is a simple, information-dense, way of naming FAs. The Δ nomenclature consists of several parts; the number of carbons, the number of desaturations, and where these are situated counting from carboxylic carbon. Linolenic acid is thus, denoted as $18{:}3\Delta^{9,12,15}$. As double bonds exist in two configurations, it is common to denote these as either *cis* or *trans*. Here all double bonds are of *cis* configuration unless explicitly stated as *trans*. Any additional functional group situated on the acyl chain, such as hydroxyl, is denoted before the acyl chain length and includes the carbon(s) where the functional group is situated, e.g. 12-OH-$18{:}1\Delta^{9}$.

In some cases, especially in a nutritional context, FAs are denoted as ω (omega), e.g. ω-3 or ω-6. Here the index number indicates the last double bond position as counted from the ω carbon in the opposite end of the carboxyl group in the acyl chain. The last double bond of $18{:}3\Delta^{9,12,15}$ is situated three carbons from the ω (Δ^{18}-Δ^{15}=3) and is thus a ω-3 FA.

Many plant lipids contain a glycerol backbone, see for example figure 1A. Stereochemical numbering (*sn*) is useful to keep track of where an enzyme performs an activity or which FA is esterified to which carbon. Glycerol-3-phosphate, for example, is esterified at the *sn-3* carbon with a phosphate, see figure 1C.

2 Introduction

Lipids are vital to plants, and their roles in plants are very diverse. Lipids are a principal constituent of cell membranes, act as signalling molecules, prevents desiccation and infections as a component of the cuticle, and enables an efficient energy and carbon storage. Lipids are not only vital to plants, but plant-derived lipids are of great importance for humans as well, as a nutrient source containing essential FAs and as feedstock for several industrial products.

The perhaps most well-known non-food application of plant oil is biodiesel, a product where FAs are methylated from seed oil or other plant sources such as tall oil and used as an environmentally friendly replacement of fossil-based diesel (Halleux *et al.*, 2008). There is, however, a multitude of other applications where plant oil-derived products are suitable as a replacement for fossil oil-based products, e.g. as lubricants, slipping agents, detergents, precursors of plastics and as a constituent of pharmaceuticals and cosmetics.

Plant seed oil consists of triacylglycerols (TAG), a glycerol backbone esterified with three FAs, see figure 1A. FAs are carboxylic acids with aliphatic chains, see figure 1B, and several industrial applications make use of the FAs rather than the oil as such. The FA composition and the stereochemical position (figure 1C) of the FAs determine the chemical and physical properties of a plant seed oil and thus its suitability in different applications (Neff & El-Agaimy, 1996). Although it may not be obvious, glycerol is a prochiral compound, and TAG species are therefore chiral, the *sn*-1 and *sn*-3 positions are thus not equivalent. Jojoba (*Simmondsia chinensis* (Link) C.K.Schneid.), a bush endemic to the Sonoran desert in North America, is the only known exemption in lipid storage in that it produces a seed oil comprised of mainly wax esters instead of TAG (Busson-Breysse *et al.*, 1994; Gentry, 1958). Wax esters are unlike TAG, molecules consisting of an FA esterified to fatty alcohol, see figure 1D. The jojoba seed oil has superior lubrication properties and is frequently used as a

constituent in cosmetics but less commonly in lubricants due to the high production costs (Shirani *et al.*, 2020).

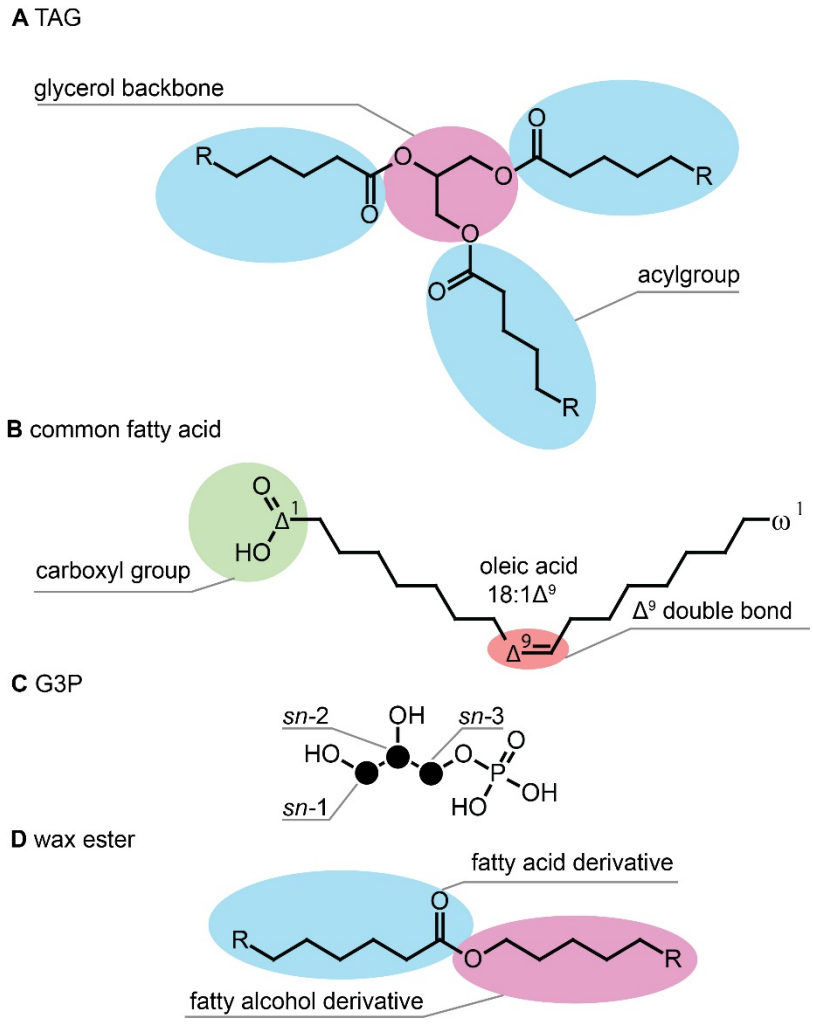

Figure 1 **A** triacylglycerol molecule with three FAs acylated to a glycerol backbone. **B** Oleic acid, a common FA with a carboxyl group at Δ^1 and a double bond at Δ^9. **C** A glycerol-3-phosphate molecule and the stereochemical numbering (*sn*) of the glycerol carbon backbone. **D** Wax ester consisting of an FA and a fatty alcohol moiety

Fatty acids fall into several different categories based on their characteristics. They may differ in acyl chain-length, degree of unsaturation, the position of desaturation(s) and additional functional groups. Most seed oils intended for human consumption such as soybean (*Glycine max* (L.) Merr.), canola (edible rapeseed oil, *Brassica napus* L.), oil palm (*Elaeis guineensis* Jacq.) and sunflower (*Helianthus annuus* L.) generally contain saturated and unsaturated FAs that are 16-18 carbons long, mainly palmitic (16:0), stearic (18:0), oleic ($18{:}1\Delta^{9}$), linoleic ($18{:}2\Delta^{9,12}$) and linolenic ($18{:}3\Delta^{9,12,15}$) acids (Dubois *et al.*, 2007). These common FAs are also the dominating FAs species in membrane lipids of most plants. Sofar, findings describe the presence of several hundred different novel or unusual FAs (UFAs) in vascular plants (Ohlrogge *et al.*, 2018). Unusual FA producing plant species, often accumulate very high levels of UFAs in their seed oil while they are often virtually absent in the plants' membrane lipids (Voelker & Kinney, 2001).

Many UFAs found in plants are suitable for a wide range of industrial applications, but economic reasons often prevent large scale production of these. Several suitable UFAs occurs in the seed oil of wild plants and are thus not fit for agriculture. Domesticated plants that produce UFAs may have additional factors that hamper large scale production.

Castor bean oil, for example, is of great interest for the oleochemical industry as it contains up to 90% of ricinoleic acid, 12-OH-$18{:}1\Delta^{9}$ (Achaya *et al.*, 1964). Industrial applications use ricinoleic acid and its derivatives as a component in a wide range of products, e.g. cosmetics, pharmaceuticals, lubricants, plasticisers, coatings and surfactants (Murphy, 1992). However, the castor bean plant, *Ricinus communis* L., does not only produce ricinoleic acid but also contains allergens and highly toxic compounds such as ricin and ricinine. These compounds cause health concerns that rightfully discourage farmers from cultivating castor bean even though it has several other advantageous agricultural traits (Ogunniyi, 2006).

Petroselinic acid ($18{:}1\Delta^{6}$) is another UFA with desirable traits for several industrial applications. Several domesticated species within Umbelliferae, e.g. carrot (*Daucus carota* subsp. *sativus* (Hoffm.) Schübl. & G. Martens) and coriander (*Coriandrum sativum* L.), produces seed oils with more than 50% of petroselinic acid (Matthaus *et al.*, 2003; Kleiman & Spencer, 1982). Domesticated varieties of both carrot and coriander are, however, adapted for other purposes than seed oil production and are currently not a very viable option for seed oil production (Iorizzo *et al.*, 2013; Diederichsen & Hammer, 2003).

Rapeseed is a domesticated oil crop which has a seed oil that contains ample amounts of a UFA, erucic acid ($22{:}1\Delta^{13}$). Erucamide, a derivative of erucic acid, is frequently used as a slipping agent and erucic acid is a highly appreciated renewable commodity (Piazza & Foglia, 2001). Rapeseed varieties bred explicitly for erucic acid production, have a seed oil with very high levels of erucic acid, exceeding 50 mol % (McVetty *et al.*, 2016). A further increase in erucic acid content is desirable not only because it results in a higher yield of erucic acid, but also that an increased concentration of erucic acid in the seed oil drastically reduces the downstream purification and production cost of erucamide (Li *et al.*, 2012; Scarth & Tang, 2006). A higher erucic acid content would thus make biobased erucamide economically more competitive against fossil oil-based options.

A robust understanding of plant lipid metabolism is essential to overcome current limitations in the production of seed oils rich in UFAs and increase the competitiveness of plant-based alternatives to fossil-based products. Lipid metabolism is still surprisingly poorly characterised, albeit their vital and diverse role in living organisms. Some processes within lipid metabolism are probably universal, whereas others differ between organisms. Plants synthesise FAs in plastids, for example, whereas animals and fungi rely on cytosolic synthesis. Even within plants, differences in lipid synthesis are apparent, as will be discussed later.

Kennedy and Weiss elucidated several enzymatic steps, central in lipid metabolism, during the late 1950s, nowadays known as the Kennedy pathway (Weiss *et al.*, 1960; Weiss & Kennedy, 1956). Biochemists have since then identified new enzymatic processes which are essential in lipid metabolism. High throughput methods of genetics and molecular biology have more recently gained a tremendous interest at the expense of more laborious classical biochemical methods. Less than half of the approximately 600 genes in Arabidopsis annotated as involved or putatively involved in lipid metabolism are confirmed *in vivo* (Allen *et al.*, 2015). Biochemical analyses are still vital for understanding lipid metabolism and has currently regained popularity. The recent identification of an acyltransferase, glycerophosphocholine acyltransferase (GPCAT), is an excellent example of where biochemistry was crucial in order to identify a gene with at the time unknown function (Gląb *et al.*, 2016).

The properties of three critical components, FA synthesis, FA modification, and TAG assembly significantly affect the composition of a seed oil during TAG synthesis. The TAG assembly requires an intricate shuttling of molecular components from different compartments of the cell. TAG synthesis also intertwines with the synthesis of several other lipids, vital for the survival of plants.

2.1 Fatty acid synthesis in plants

Most eukaryotic organisms synthesise FAs in the cytosol, but plants differ in this regard. Instead, plants synthesise *de novo* FAs in the stroma of plastids (Harwood, 1996; Ohlrogge *et al.*, 1979). Acetyl-CoA carboxylase (ACC) facilitates the formation of malonyl-CoA from bicarbonate and acetyl-CoA (2:0-CoA), and this initiates the dedicated synthesis of FAs (Kannangara & Stumpf, 1972).

The plastidial ACC in most plants are of prokaryotic origin. Grasses, however, possess a plastidial ACC of eukaryotic origin (Konishi *et al.*, 1996). Aryloxyphenoxypropionate and cyclohexanedione are two chemical compounds that efficiently inhibit the eukaryotic ACC of grasses, and thus FA synthesis. The inhibition of FA synthesis is detrimental to plants and these and other similar compounds act as efficient grass specific herbicides (Burton *et al.*, 1989). The loss of prokaryotic plastidial ACC in grasses was the first published example of a eukaryotic nucleus residing genes replacement of a plastidial gene (Konishi *et al.*, 1996).

The malonyl moiety produced by ACC is subsequently transferred from CoA to an acyl carrier protein (ACP) by malonyl-CoA:acyl carrier protein malonyltransferase (MCMT) (Guerra & Ohlrogge, 1986). Long-chain FAs are synthesised cyclically by fatty acid synthase (FAS), an enzymatic complex, where malonyl-ACP provides two carbon for elongation in each cycle (Harwood, 1988). Each cycle in FAS includes several steps; condensation, reduction, dehydration, and reduction, see figure 2 (Harwood, 1988). β-ketoacyl-ACP synthases (KAS) catalyses the condensation in FAS. Plants have several isoforms of KAS, where KAS III catalyses the initial condensation of acetyl-CoA and malonyl-ACP, while the other involved KAS utilise acyl-ACP and malonyl-ACP as substrates (Slabas & Fawcett, 1992). KAS I catalyses the condensation of acyl chains up to 16 carbons length whereas KAS II carries out the condensation of palmitoyl-ACP (16:0-ACP) and malonyl-ACP to β-keto-stearoyl-ACP (Slabas & Fawcett, 1992). The KAS enzymes catalyse several

chemical reactions included in a Claisen condensation, see figure 3 (Huang *et al.*, 1998). In most plant species this cyclic elongation of acyl-ACP carries on until the acyl chains are 16 to 18 carbons long. A plastidial residing desaturase, Δ^9 stearoyl-ACP desaturase (SAD), introduce a double bond in 18:0-ACP and thus desaturate it, which results in 18:1-Δ^9-ACP, see figure 4 for an overview of plastidial processes during FA synthesis (McKeon & Stumpf, 1982).

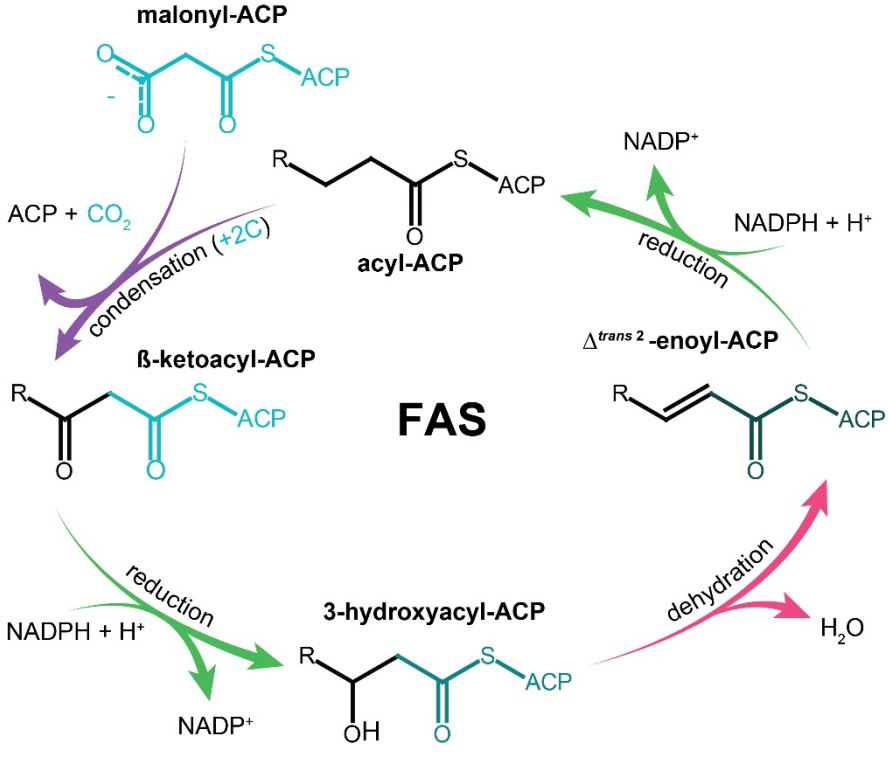

Figure 2 schematic overview of the cyclic reactions catalysing acyl elongation in FAS. FAS use malonyl-ACP for two carbon elongation during each cycle. The initial condensation catalysed by KASII uses acetyl-CoA instead of acyl-ACP. The condensed β-ketoacyl-ACP is further reduced into 3-hydroxy acyl-ACP and dehydrated into Δ^{trans2}-enoyl-ACP. A final reduction results in acyl-ACP, which in turn can be further elongated by KAS I and later KAS II.
Abbreviations: **ACP –** acyl carrier protein, **FAS** – fatty acid synthase

Removal of the acyl group from ACP terminates the FA synthesis; either through transacylation or release of free FAs (FFAs) (Ohlrogge & Browse, 1995). Acyl-ACP thioesterase FatA and FatB facilitate the hydrolysis of FFAs from acyl-ACP (Jones, 1995). The vast majority of FAs released by the thioesterases in Arabidopsis are 18:1Δ^9, followed by minor amounts of 16:0 and 18:0 (Li-Beisson *et al.*, 2010).

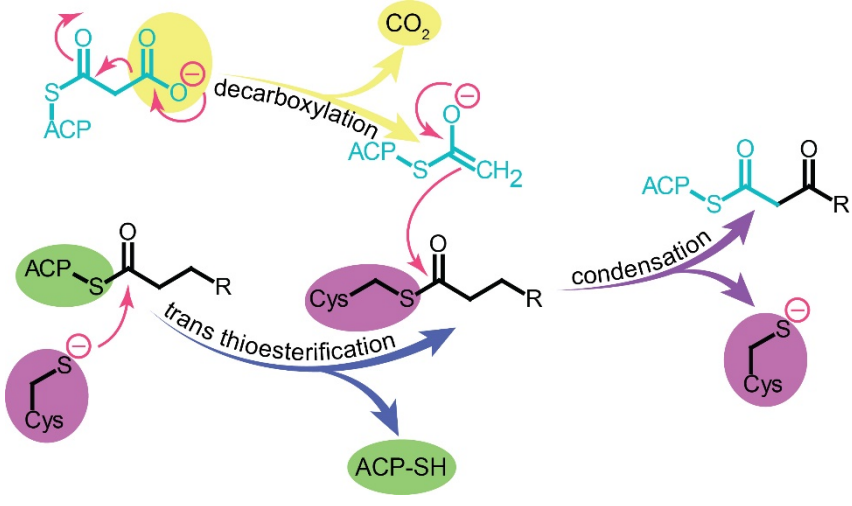

Figure 3 overview of the reactions catalysed by the KAS enzymes during the condensation in FAS. Malonyl-ACP is decarboxylated while the acyl-ACP (or acetyl-CoA) is transferred to a Cys residue of a KAS enzyme. The acyl is later condensed to the enolate ion resulting in a β-ketoacyl-ACP. ***Abbreviations:*** **ACP** – acyl carrier protein, **Cys** - cystine

Transcriptional factors such as WRI1 rigidly control the gross rate of FA synthesis (Grimberg *et al.*, 2015). WRI1 autoregulates itself through a negative feedback loop, which may act as a fail-safe and may enable a faster response time than otherwise possible (Snell *et al.*, 2019). These features potentially allow tuning of the FA synthesis rate. Several post-translational regulations are known to affect FA synthesis as well. ACC which facilitates the first committed step in FA synthesis, for example, is inhibited by 18:1Δ^9-ACP, the primary end product in FA synthesis. Further, light, energy level and substrate availability regulates the ACC activity and thus enable a fine-tuning of FA synthesis (Troncoso-Ponce *et al.*, 2016).

2.2 Export of acyl-chains from the plastid

The nascent FFAs released from FAS are available for export to other subcellular compartments. Reports describe several processes that facilitate the export of FFAs, but the full process is yet not fully clarified and understood. Several enzymes involved in the acyl export from the plastid are known. Studies of an Arabidopsis transporter protein, fatty acid export 1 (FAX1), situated in the inner envelope of plastids suggest that FAX1 is involved in the transfer of FFA past membranes (Li *et al.*, 2015). Studies also indicate the presence of long-chain acyl-CoA synthetases (LACS) on the surface of plastids that drives the export. LACS enzymes catalyse the formation of acyl-CoA from FFA and coenzyme A (CoA). Several LACS genes are present in Arabidopsis and LACS9, located in the plastid envelope is a significant contributor to acyl-CoA formation in purified plastids (Schnurr, 2002). An additional LACS enzyme, LACS1, localised in the endoplasmic reticulum (ER) also have an overlapping function with LACS9 (Zhao *et al.*, 2010). LACS9, LACS1 and FAX1 are, however, not sole in the export of nascent FAs; other yet unknown concurrent processes facilitate the export of FAs from the plastid as well. The exported acyl-CoAs are then available, for glycerolipid assembly and other processes in the ER.

Rapid kinetic labelling of Arabidopsis cell suspension cultures with [^{14}C]acetate reveals a surprisingly short lag time between incubation and synthesis of radiolabelled phosphatidylcholine (PC). A hypothesis that explains such a short lag time includes channelling of acyl-CoA from LACS more or less instantaneously to PC via lysophosphatidylcholine acyltransferase (LPCAT) (Tjellström *et al.*, 2012). LPCAT is an enzyme that catalyses the formation of PC from lysophosphatidylcholine (LPC) and acyl-CoA. For more details on this phenomenon, see pages 26-29.

Generally, ER is the subcellular organelle ascribed for LPCAT localisation, but a few studies also suggest a plastidial localisation in leek (*Allium porrum* L.) and pea (*Pisum sativum* L.) (Kjellberg *et al.*, 2000; Bessoule *et al.*, 1995). An extensive network of ER surrounds chloroplasts in Arabidopsis, and several membrane contact sites are apparent (Andersson *et al.*, 2007). Protein-protein interactions probably maintain these contact sites. The observed LPCAT activity in the plastids may thus be contamination of remnant membranes from ER or ER localised proteins. Thus, LPCAT may be present in the ER, or both in plastids and ER. In any case, it is at least associated with contact sites between plastids and ER. There are strong indications that LPCAT mediated transfer of FAs between plastids and ER occurs irrespective of LPCAT's actual subcellular membrane localisation.

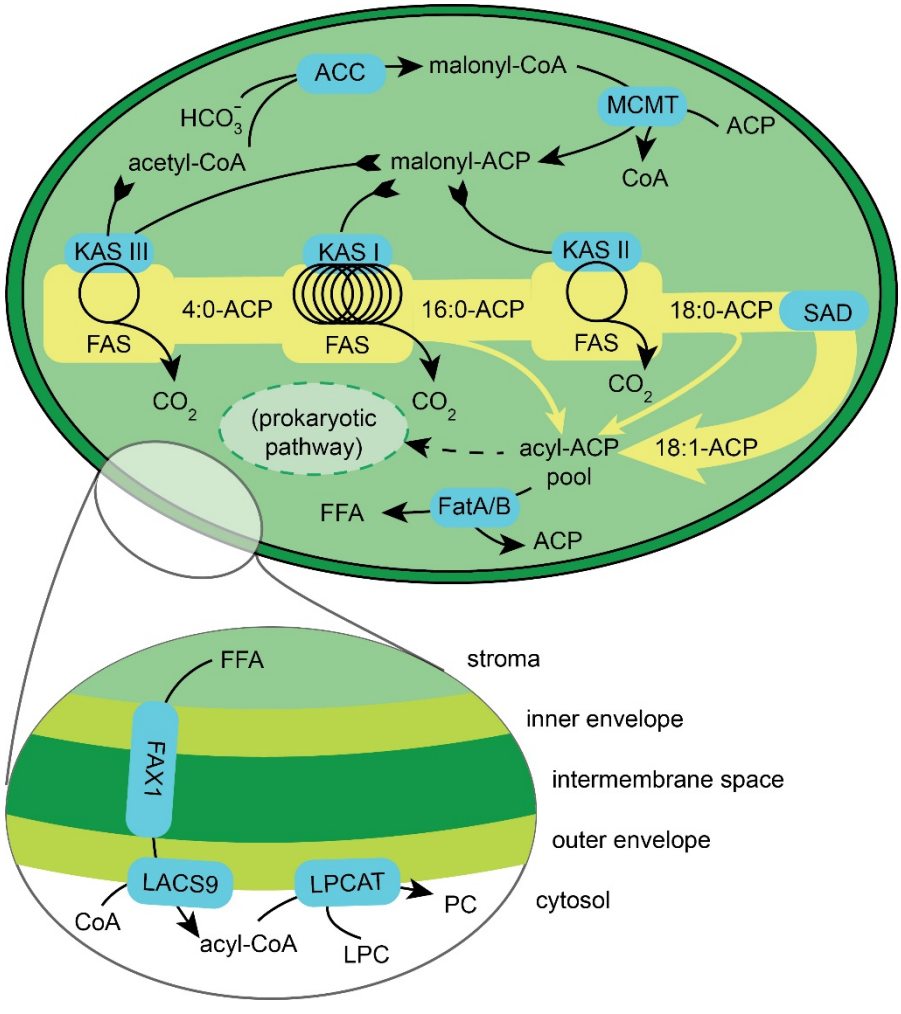

Figure 4 overview of the synthesis of FAs in the plastid and the following export to the cytosol through proposed mechanisms. ACC produces malonyl-CoA from HCO_3^- and acetyl-CoA. The malonyl-CoA is later thioesterified to ACP. The first condensation catalysed by KAS III use malonyl-ACP and acetyl-CoA and results in 4:0-ACP after the first cycle of FAS. The FAS complex further elongates 4:0-ACP into acyl chains of up to 18 carbons length in a cyclic manner. KAS I facilitate the condensing reaction from 4:0-ACP to 16:0-ACP, while KAS II carries out the final condensation of 16:0-ACP to 18:0-ACP. SAD desaturate a large proportion of the nascent 18:0-ACP into 18:1-ACP. The elongated acyl chains are later used either by the prokaryotic lipid assembly which occurs in 16:3 plant species or released as FFA by FatA and FatB. FAX1 facilitates the transfer of FFA across the plastidial membranes where the FFA are thioesterified with CoA by LACS9. LPACT may play an

essential role in many plant species and incorporate the nascent acyl-CoAs into PC. ***Abbreviations:*** **CoA** – coenzyme A, **ACC** - acetyl-CoA carboxylase, **MCMT** - malonyl-CoA:acyl carrier protein malonyltransferase, **ACP** – acyl carrier protein, **KAS(I, II & III)** - β-ketoacyl-ACP synthase, **FAS** - fatty acid synthase, **SAD** - Δ^9 stearoyl-ACP desaturase, **FatA/B** – thioesterase, **FFA** – free fatty acid, **FAX1** – fatty acid export protein 1, **LACS9** - Long-chain acyl-CoA synthetase 9, **LPCAT** - lysophosphatidylcholine acyltransferase, **LPC** – lysophosphatidylcholine, **PC** - phosphatidylcholine

2.3 TAG assembly in the endoplasmic reticulum

In developing oilseeds, a significant proportion of the nascent FAs end up as a constituent of TAG. Assembly of glycerolipids such as TAG in ER is dependent on FAs produced and exported from the plastids. TAG is produced in the ER by a range of acyltransferases and other enzymatic processes, see figure 5 for an overview. Diacylglycerol (DAG) is the final precursor of TAG, and the Kennedy pathway is a metabolic route that gives rise to *de novo* synthesised DAG subsequently converted into TAG (Weiss *et al.*, 1960; Weiss & Kennedy, 1956). All known acyltransferases in the Kennedy pathway utilises acyl-CoA as acyl-donor. The first step in the Kennedy pathway is the acylation of the *sn*-1 position of glycerol-3-phosphate (G3P, figure 1C) carried out by glycerol-3-phosphate acyltransferase (GPAT), which results in lysophosphatidic acid (LPA) (Zheng, 2003). LPA is further acylated into phosphatidic acid (PA) at the *sn*-2 position by lysophosphatidic acid acyltransferase (LPAT) (Knutzon *et al.*, 1995). Phosphatidic acid phosphatase (PAP) subsequently decouples the phosphate group at *sn*-3 and converts PA into DAG (Eastmond *et al.*, 2010). DAG is besides being the precursor of TAG, also a precursor of PC and other phospholipids. CDP-choline:DAG cholinephosphotransferase (CPT) produces *de novo* PC from DAG and CDP-choline (Dewey *et al.*, 1994).

PC is of central interest in TAG assembly, as many acyl desaturations and other FA modifications occur while acyl-chains are associated with PC. Further, there are indications as previously mentioned that most nascent FA exported from the plastid might not always enter lipid assembly directly through acyltransferases in the Kennedy pathway but initially though the acyl modification cycle of PC. The modification cycle of PC facilitates incorporation, modification and release of FAs from PC. Several enzymes incorporate FAs into PC in a range of different ways besides the previously described CPT. Phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), for example, interconvert DAG and PC but gives no net gain of PC (Lu *et al.*, 2009). The forward reaction of LPCAT incorporates FAs from the acyl-CoA pool at the

sn-2 position of LPC. LPC, in its turn, may be synthesised by another acyltransferase, GPCAT, which catalyses the formation of LPC from glycerol-3-phosphocholine (GPC) and acyl-CoA (Gląb *et al.*, 2016).

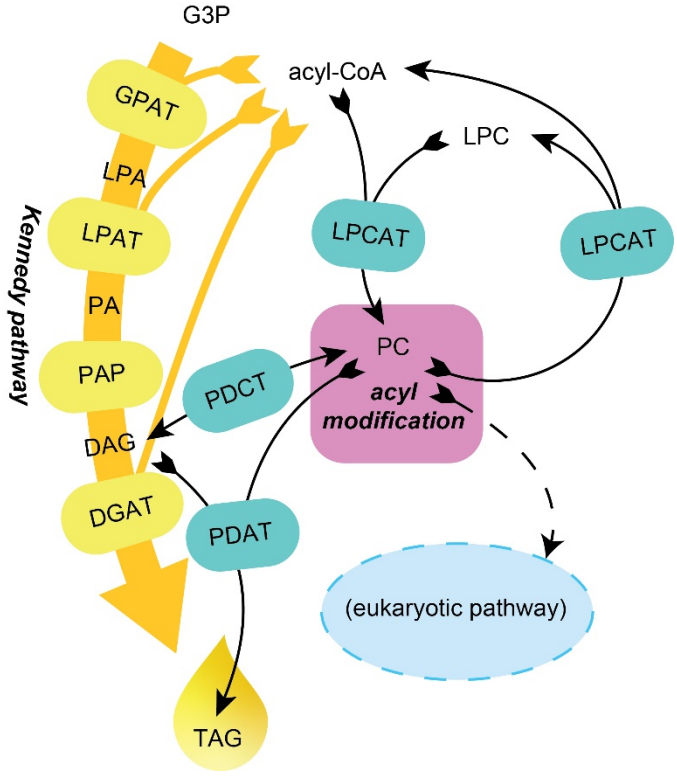

Figure 5 illustrates a simplified overview of TAG assembly in the ER. The Kennedy pathway includes the sequential acylation of G3P into TAG. GPAT acylates the *sn*-1 position of G3P with an acyl group derived from acyl-CoA into LPA which in turn is similarly acylated by LPAT into PA. PAP removes the phosphate group at *sn*-3 and converts the PA into DAG. DAG is a critical branch point in lipid metabolism since the production of both TAG and *de novo* synthesised PC utilises DAG. DGAT catalyses the final acylation at *sn*-3, which results in TAG. PDAT is also concurrently promoting the acyl-CoA independent synthesis of TAG, recruiting an acyl moiety from PC which transesterifies DAG into TAG. Acyl groups associated with PC are often modified, which is an essential feature in many plants' lipid metabolism. Acyl groups can enter PC through several processes. PDCT interconverts the phosphocholine headgroup between PC and DAG enabling acyl groups from DAG to enter PC for modification. LPCAT enables rapid incorporation of acyl

groups into PC via the acylation of LPC and the reverse reaction of LPCAT releases potentially PC modified acyl-CoAs into the acyl-CoA pool. The eukaryotic pathway also utilises PC derived intermediates for the synthesis of thylakoid membrane lipids. ***Abbreviations:*** **G3P** - glycerol-3-phosphate, **CoA -** coenzyme A, **GPAT** - glycerol-3-phosphate acyltransferase, **LPA** - lysophosphatidic acid, **LPAT**- lysophosphatidic acid acyltransferase, **PA** - phosphatidic acid, **PAP** - phosphatidic acid phosphatase, **DAG** – diacylglycerol, **DGAT** - acyl-CoA:diacylglycerol acyltransferase, **TAG** – triacylglycerol, **LPC** – lysophosphatidylcholine, **LPCAT** - lysophosphatidylcholine acyltransferase, **PC** – phosphatidylcholine, **PDCT** - phosphatidylcholine diacylglycerol cholinephosphotransferase, **PDAT** - phospholipid: diacylglycerol acyltransferase

Several important modifications of FA occurs, and many UFAs gain their functional groups, while associated with PC. Several of the examples described below on page 35, figure 7, which illustrate modifications of FAs into UFAs while they are a moiety of PC.

As with the incorporation of FAs into PC, multiple pathways facilitate the release of FAs from PC. The reverse reaction of LPCAT releases FAs to the acyl-CoA pool (Lager *et al.*, 2013). Phospholipase C and D, PDCT and potentially also the reverse reaction of CPT, facilitate the incorporation of FAs into substrates used in the Kennedy pathway for TAG synthesis (Bates *et al.*, 2013; Slack *et al.*, 1985). Further, another acyltransferase, phospholipid: diacylglycerol acyltransferase (PDAT), produces TAG through transacylation of acyl groups from PC to DAG (Dahlqvist *et al.*, 2000).

Metabolic flux analyses of *de novo* synthesised FAs reveal that plants may have different strategies to incorporate nascent FA into glycerolipids as previously mentioned. The flux of nascent FAs in developing soybean embryos, for example, is far higher into the acyl modification cycle than into *de novo* lipid assembly via the Kennedy pathway (Bates *et al.*, 2009). The soybean seed oil reflects the high flux of nascent FAs into PC modification cycle, where a majority of the FAs are of PC modified origin, such as the polyunsaturated FAs $18{:}2\Delta^{9,12}$ and $18{:}3\Delta^{9,12,15}$. Studies reveal similar results in several other species, e.g. safflower (*Carthamus tinctorius* L.), flax (*Linum usitatissimum* L.), and Arabidopsis (Bates & Browse, 2012; Griffiths *et al.*, 1988; Slack *et al.*, 1978). These are all species that boast a TAG FA composition that is rather high in FAs modified in the PC modification cycle. Other species, e.g. avocado (*Persea americana* Mill.), cigar flower (*Cuphea ignea* A. DC.), and cacao tree (*Theobroma cacao* L.) are species with a relatively low content of FAs edited during the PC modification cycle in their seed oil (Griffiths & Harwood, 1991).

Studies of these species indicate that these are more likely to incorporate the nascent FAs in the Kennedy pathway directly instead of through the PC modification cycle (Griffiths & Harwood, 1991; Bafor *et al.*, 1990; Griffiths *et al.*, 1988). Interestingly, coriander, containing high levels of UFAs not modified while associated with PC, still incorporate most UFAs into PC before TAG synthesis (Cahoon & Ohlrogge, 1994). In conclusion, these studies reveal that different plants rely on different routes for incorporating nascent FAs into glycerolipid synthesis.

Acyltransferases facilitate the final acylation of TAG regardless of which route that gives rise to the DAG available for TAG synthesis, as seen in figure 5. PDAT, as mentioned earlier, facilitates acyl transfer from PC to DAG, while acyl-CoA:diacylglycerol acyltransferase (DGAT) facilitates TAG formation via the Kennedy pathway. DGAT is the sole enzyme in the Kennedy pathway exclusively directed to TAG assembly and utilises acyl-CoA as acyl-donor, unlike PDAT.

DGAT is of great importance, both quantitatively and qualitatively in TAG synthesis (Shockey *et al.*, 2006). As of yet, researchers have identified at least three types of DGAT. DGAT1 first identified in mice, and DGAT2 first cloned from an oleaginous fungus, and both are present in plants (Lardizabal *et al.*, 2001; Cases *et al.*, 1998). DGAT2 does not seem to contribute to TAG accumulation in developing seeds of Arabidopsis, whereas DGAT1 null mutants exhibit a decreased TAG accumulation (Zhang *et al.*, 2009; Routaboul *et al.*, 1999; Katavic *et al.*, 1995). Consistent with these findings, overexpression of DGAT1 in Arabidopsis during seed development enhances the amount of TAG deposition in seeds (Jako *et al.*, 2001). The DGAT2 of Arabidopsis is, however, an active catalytic DGAT enzyme and exhibits a substrate specificity that is different from that of the DGAT1 form (Ayme *et al.*, 2014; Zhou *et al.*, 2013). DGAT2 enzymes appear to be involved in the incorporation of UFAs into TAG in plant species expressing high levels of DGAT2 in their seeds. The DGAT2 enzymes of the tung tree (*Vernicia fordii* (Hemsl.) Airy Shaw), castor bean and ironweed (*Vernonia galamensis* (Cass.) Less.) are all capable of introducing UFAs into seed oil (Li *et al.*, 2010a; Burgal *et al.*, 2008; Shockey *et al.*, 2006).

Both DGAT1 and DGAT2 enzymes are membrane-bound, located in the ER (Shockey *et al.*, 2006). DGAT1 and DGAT2 differ from each other in many regards, albeit their related catalytic activity. The two classes distinguish from each other in membrane-topology, amino acid length, and they belong to different protein families (Turchetto-Zolet *et al.*, 2011; Yen *et al.*, 2008). The

third class of DGAT present in plants, DGAT3, is soluble and is yet to be confirmed to be associated with seed oil accumulation (Saha *et al.*, 2006). The three classes of DGAT are probably the result of convergent evolution and share no significant sequence homology (Aymé *et al.*, 2018).

The TAG composition in oilseeds is hence, dependent on the abundance of the various acyltransferases, as well as their specificity towards acyl-donors and acyl-acceptors and the substrate availability and composition. The final acylation of DAG, which results in TAG, is facilitated by either DGAT or PDAT, and these are the final determinants of the TAG composition. PDAT and DGAT may concurrently contribute to the accumulating TAG pool. Their relative contribution differs not only between different plant species, but temporal and spatial variations co-exist as well (Li *et al.*, 2010b). Spatial differences in TAG composition is present, not only within a single seed, but lipid composition heterogenicity is also present at tissue-level (Sturtevant *et al.*, 2016).

It is crucial to keep in mind that many other lipids beside TAG, are assembled in the ER. Both PA and DAG produced in the Kennedy pathway are precursors for several other lipids, phosphatidylinositol (PI), phosphatidyglycerophosphate (PGP) and PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), respectively. Further, FAs from PC are also substrates for the synthesis of thylakoid membrane lipids.

2.4 Acyl import to the plastids

Acyl groups are not only exported from the plastids to the ER as recently described, but FAs are, also, imported from the ER to the plastid. Thylakoid membranes within plastids consist mainly of the galactolipids, monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) and the sulfolipid sulfoquinovosyl diacylglycerol (SQDG) (Dorne *et al.*, 1990). These are lipids synthesised by two separate pathways, the prokaryotic and the eukaryotic (Heinz & Roughan, 1983). Plants fall into two categories; those that contain $16{:}3\Delta^{7,10,13}$, e.g. spinach (*Spinacia oleracea* L.) and Arabidopsis, hence called 16:3 species while those devoid of $16{:}3\Delta^{7,10,13}$ in photosynthetic tissues are called 18:3 plants, reflecting a mix of prokaryotic and eukaryotic and only eukaryotic pathways, respectively. The prokaryotic pathway depends on *de novo* synthesised FAs recruited from the plastidial acyl-ACP pool, while the eukaryotic pathway utilises FAs first exported to the ER and then returned to the plastid. The individual LPAT specificity within these two pathways produces a

hallmark that differentiates the lipids synthesised by either pathway (Roughan & Slack, 1984). The plastidial LPAT specificity favours acylating saturated acyl-chains of 16 carbons length while the ER located LPAT favours unsaturated 18 carbon acyl-chains. Lipids synthesised through the eukaryotic pathway, therefore, contain an 18 carbon acyl group at the *sn*-2 position while those from the prokaryotic contains a 16 carbon acyl group. The FAs in lipids produced by the eukaryotic pathway are, thus, synthesised in plastids and exported to the ER. In the ER these FAs are associated with PC, desaturated and reintroduced to the plastid, potentially further desaturated and used for assembly of thylakoid membrane lipid species as depicted in figure 6.

About 12 % of the angiosperms produce MGDG, DGDG, and SQDG through both pathways, while the majority seems to rely on the eukaryotic pathway alone (Mongrand *et al.*, 1998). There are strong indications that a transporter system, constituted by the TGD1-5 proteins, facilitate the reintroduction of PC derived FAs into plastids in Arabidopsis (Karki *et al.*, 2019). It is still however unclear which PC derived intermediate that is transported by this complex (Karki *et al.*, 2019).

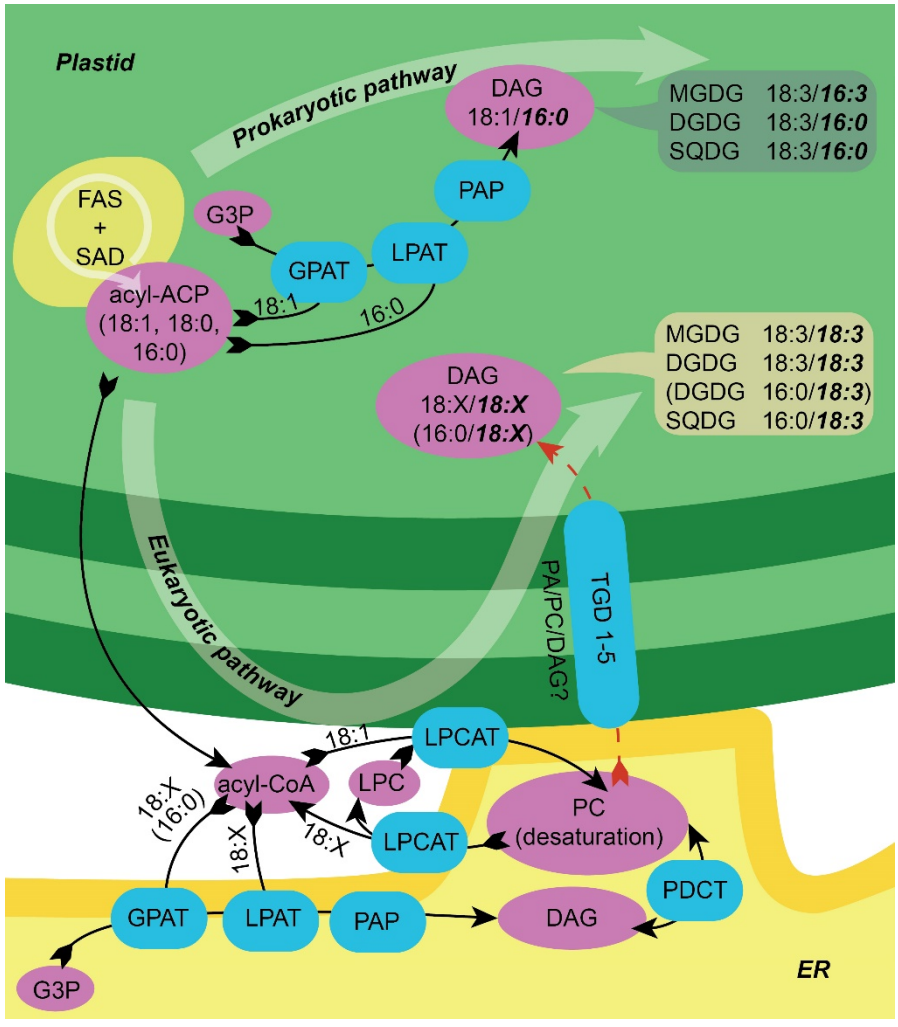

Figure 6 is an overview of two alternative metabolic routes present in Arabidopsis for the synthesis of thylakoid membrane lipids. The prokaryotic pathway use acyl-ACP substrates, where the substrate specificities of GPAT and LPAT generate *sn*-1-18:1-*sn*-2-16:0-DAG. Thylakoid membrane lipids derived from the prokaryotic pathway therefore always contain a 16:X acyl moiety at *sn*-2. The specificity of the eukaryotic acyltransferases, on the other hand, give rise to PC species that exclusively include an 18:X acyl moiety at *sn*-2. These are imported to the plastid by the transporter system TGD1-5. Thylakoid membrane lipids made from the eukaryotic pathway will thus have an 18:X acyl moiety at *sn*-2 instead of 16:X. ***Abbreviations***: **FAS** – fatty acid synthase, **SAD** - Δ9 stearoyl-ACP desaturase, **ACP** – acyl carrier protein, **G3P**

- glycerol-3-phosphate, **GPAT** - glycerol-3-phosphate acyltransferase, **LPAT**- lysophosphatidic acid acyltransferase, **PAP** - phosphatidic acid phosphatase, **DAG** – diacylglycerol, **MGDG** - monogalactosyl diacylglycerol, **DGDG** - digalactosyl diacylglycerol, **SQDG** - sulfoquinovosyl diacylglycerol, **CoA** – coenzyme A, **LPC** – lysophosphatidylcholine, **LPCAT** - lysophosphatidylcholine acyltransferase, **PC** – phosphatidylcholine, **PDCT** - phosphatidylcholine diacylglycerol cholinephosphotransferase, **PA** - phosphatidic acid, **TGD(1-5)** – trigalactosyldiacylglycerol, **ER** – endoplasmic reticulum

2.5 Unusual fatty acid modifications

Plants can produce several hundreds of different UFAs. Modification of FAs into UFAs occurs at several different stages during FAs synthesis and lipid assembly.

Several UFAs are synthesised directly in connection to the fatty acid synthesis in the plastids. California bay, *Umbellularia californica* (Hook. & Arn.) Nutt., is a species known to accumulate high levels of medium-chain fatty acids (MCFA) (Hopkins et al., 1966). Transgenic canola (*Brassica napus* L.) expressing *FatB1* from California bay exhibit a significantly altered FA composition in the accumulated seed oil with drastically increased levels of laurate (12:0), which increases from 0.02 mol% in the wildtype to up to 60 mol% in transgenic lines (Voelker et al., 1996).

Another species with high levels of MCFA in its seed oil is Wright's waxweed, *Cuphea wrightii* A. Gray (Thompson & Kleiman, 1988). *KAS A1*, as well as *FatB1* and *FatB2* from *C. wrigthii*, have been cloned and expressed in transgenic Arabidopsis. Expression of either *CwFatB1* or *CwFatB2* enhances levels of MCFA (10:0 and 12:0) in the Arabidopsis seed oil while the expression of CwKAS A1 does not. However, if either of the two *CwFatB*s is co-expressed with *CwKAS A1*, a further significant increase of 10:0 and 12:0 in the seed oil is observed. The additional increase of MCFA indicates that efficient production of MCFA may not only depend on fatty acid thioesterases alone but also, on specialised KAS enzymes during FA synthesis, see figure 4 for context (Leonard *et al.*, 1998).

Many plant species contain plastidial acyl-ACP desaturases similar to the previously mentioned Δ^9 SAD. These acyl-ACP desaturases are in several cases capable of utilising palmitoyl-ACP instead of stearoyl-ACP and introduces double bonds at novel positions compared to SAD. Unusual FAs produced through such deviating acyl-ACP desaturases include Δ^6 palmitic acid (16:1Δ^6)

in black-eyed Susan vine, *Thunbergia alata* Bojer ex Sims (Cahoon *et al.*, 1994) and Δ^4 palmitic acid ($16{:}1\Delta^4$) in coriander (Cahoon & Ohlrogge, 1994). Δ^4 palmitic acid is the precursor of petroselinic acid, which coriander produces through subsequent elongation of Δ^4 palmitoyl-ACP into petroselinoyl-ACP ($18{:}1\Delta^6$). Petroselinic acid with its deviating double bond position is suitable as a precursor of adipic acid (6:0 dicarboxylic), a widely used monomer for polymer production, and 12:0, a common constituent of surfactants and detergents (Cahoon *et al.*, 1992).

A wide range of enzymes that modify FAs into UFAs is associated with the ER. The common FAs $18{:}2\Delta^{9,12}$ and $18{:}3\Delta^{9,12,15}$, are desaturated while acylated at *sn*-2 of PC during the PC modification cycle. Fatty acid desaturase 2 (FAD2) desaturate $18{:}1\Delta^9$ at Δ^{12} into $18{:}2\Delta^{9,12}$ while FAD3 catalyse the further desaturation of $18{:}2\Delta^{9,12}$ into $18{:}3\Delta^{9,12,15}$. FAD2 enzymes contain a conserved motif which is vital for enzymatic activity. The FAD2 motif consists of eight conserved histamine residues in three histamine boxes (Shanklin & Cahoon, 1998). This motif is also present in FAD2 from several organisms belonging to other phyla as well. The sequence homology and the FAD2 motif has aided in search of a wide range of FAD2-like enzymes responsible for modifications that produce UFAs (Cahoon & Kinney, 2005).

Castor bean produces high levels of 12-OH-$18{:}1\Delta^9$ in its seed oil. The Δ^{12} hydroxylase (FAH12) that converts oleate into ricinoleate in castor bean and the desaturation of $18{:}1\Delta^9$ into $18{:}2\Delta^{9,12}$ catalysed by FAD2 share not only the modification on the same delta position but also the substrate, an oleoyl moiety of PC. Functional characterisation of castor bean FAD2-like cDNA in transgenic plants confirms that this FAD2-like enzyme, FAH12, indeed catalyses the hydroxylation of oleate in castor bean (Van De Loo *et al.*, 1995). Several other UFAs, e.g. vernolic acid (12,13-epoxy-$18{:}1\Delta^9$), crepenynic acid (12-alkyne-$18{:}1\Delta^9$), and several conjugated FAs (polyunsaturated FAs with conjugated double bonds, e.g. α-eleostearic acid, $18{:}3\Delta^{9,trans11,trans13}$) are all modified by FAD2-like enzymes, see figure 7 (Dyer *et al.*, 2002; Cahoon *et al.*, 2001; Lee *et al.*, 1998). Many of these UFAs are of industrial interest. FAD2-like enzymes modify FAs into UFAs while associated with PC, but the PC pool contains minor levels of these UFAs. The UFAs modified by FAD2-like enzymes are efficiently removed from PC and rapidly incorporated into TAG (Bafor *et al.*, 1991; Moreau & Stumpf, 1981).

Accumulating evidence indicates that yet another form of acyl desaturases exists in plants. These desaturases may utilise acyl-CoA instead of acyl-ACP or glycerolipids such as PC. Acyl-CoA desaturase-like genes (*ADS*) encode these desaturating enzymes which resemble those found in mammals and yeast (Smith *et al.*, 2013). Meadowfoam, *Limnanthes alba* Benth. and several closely related species produce an oil quality that contains an eicosenoic acid with an unusual desaturation position, 20:1Δ^5. Time course studies using radioactive substrates and microsomal preparations from developing seeds of meadowfoam reveal that this unusual desaturase activity is probably utilising acyl-CoA as substrate (Moreau *et al.*, 1981). An expressed *ADS* gene cloned from Douglas meadowfoam, *Limnanthes douglasii* R. Br., desaturates a range of FAs at the unusual Δ^5 position in somatic embryos of soybean (Cahoon *et al.*, 2000). *Anemone* L. is another genus that contains an example of an *ADS* encoded enzyme that potentially utilises acyl-CoA. Sciadonic acid (20:3$\Delta^{5,11,14}$) represent nine per cent of the FAs in the seed oil of *Anemone rivularis* Buch.-Ham. ex DC. var. *rivularis*. Transgenic Arabidopsis expressing two *A. rivularis* var. *rivularis* derived *ADS* genes produce significant amounts of sciadonic acid. Further, there are strong indications that these enzymes indeed utilise acyl-CoA as substrate (Sayanova *et al.*, 2007). Not all enzymes encoded by *ADS* genes in plants utilise acyl-CoA however, as at least one Arabidopsis *ADS* gene, At*ADS3*, encodes for FAD5 which catalyses the Δ^7 desaturation on the 16 carbon *sn*-2 moiety of MGDG in Arabidopsis (Heilmann *et al.*, 2004).

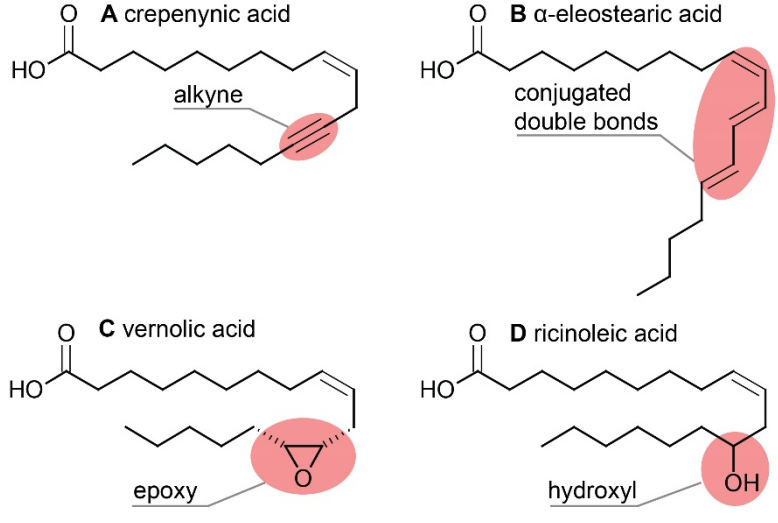

Figure 7 structural features of several UFAs, unusual features are higligthed in red. **A** Crepenyic acid **B** α-eleostearic acid, **C** vernolic acid and **D** ricinoleic acid

Many plants produce seed oils containing FAs that are longer than that produced by plastidial FAS. Elongation beyond the acyl-chain lengths provided by the plastidial FAS results in very long-chain fatty acids (VLCFA), such as $22{:}1\Delta^{13}$, abundant in many species within Brassicales. The elongase, a membrane-bound enzyme-complex which closely resembles that of FAS in the plastid, elongate FA into VLCFA (von Wettstein-Knowles, 1982). The initial step of elongation catalysed by β-ketoacyl-CoA synthase (KCS) condensate malonyl-CoA and a long-chain acyl-CoA into β-ketoacyl-CoA. The elongase complex further reduces and dehydrates the β-ketoacyl-CoA, which results in a two carbon elongation of the initial long-chain acyl-CoA.

Albeit several similarities between the FAS and elongase complex, see figure 2, they differ in a few aspects. The ER localised elongase complex utilise moieties associated with CoA while the plastidial FAS requires that at least one of the substrates is a moiety of ACP. Both the KAS enzymes that facilitate condensation in the FAS and the KCS of the elongase complex belong to the thiolase superfamily. In FAS, KASIII catalyses the initial condensation and is the only condensing enzyme that utilises acetyl-CoA as substrate. Both KCS and KAS III share the same active site architecture, which is different from that of KAS I and KAS II. Phylogenetic analyses also indicate that KCS and KAS III are more similar to each other than KAS III is to the KAS I and KAS III (Jiang *et al.*, 2008).

The *FAE1* gene in Arabidopsis encodes a seed-specific KCS responsible for the condensation initiating the elongation of FA into VLCFA. Studies reveal that the activity of KCS is rate-limiting in the elongase complex. The KCS enzyme also determines the end product acyl length through its acyl-CoA specificity (Millar & Kunst, 1997). The identified *FAE1* homologues of *T. majus* (Mietkiewska *et al.*, 2004) and *Crambe hispanica* subsp. *abyssinica* (Hochst. ex R.E. Fr.) Prina (Mietkiewska *et al.*, 2007) are capable of initiating the elongation of various acyl-CoA into erucic acid, which is a significant FA in these species seed oils.

The recent identification of two UFAs, nebraskanic acid 7,18-OH-$24{:}1\Delta^{15}$ and wuhanic acid 7,18-OH-$24{:}2\Delta^{15,21}$, and the following elucidation of the biosynthetic synthesis of these, presents yet another mechanism, for the synthesis of UFAs (Li *et al.*, 2018). Nebraskanic and wuhanic FAs comprise up to 50 wt% of the seed oil from the Brassicaceae species *Orychophragmus violaceus* (L.) O.E. Schulz. Several genes identified in *O. violaceus* are most likely involved in the synthesis of these VLCFAs that contains two hydroxyl

groups at Δ^7 and Δ^{18}. The synthesis of both these FAs starts with an 18 carbon moiety associated with PC and includes a Δ^{12} hydroxylase and the following elongation process, which also introduces the second hydroxyl group. An identified *FAD2*-like gene, Ov*FAD2-2*, encodes for an enzyme that contains an amino acid substitution situated before the first histamine box known to occur in FAD2-like enzymes, e.g. the castor bean FAH12, which catalyses the introduction of hydroxyl groups at Δ^{12} of FAs (Cahoon & Kinney, 2005). Monohydroxy FAs, e.g. 12-OH-18:1Δ^9 and 12-OH-18:2$\Delta^{9,15}$, are present in the seed oil of transgenic Arabidopsis expressing *OvFAD2-2*, but the seed oil is still devoid of dihydroxy FA (Li *et al.*, 2018). An elongase gene, *OvFAE1-1*, encoding for a divergent KCS enzyme is capable of condensing the monohydroxy 18 carbon FAs with malonyl-CoA. Reduction of the resulting 3-ketotacyl-CoA into 3-hydroxyacyl-CoA seems to occur as in the generic elongation process catalysed by the elongase complex. At this point, the divergent OvFAE1-1 enzyme probably discontinues the following processes occurring by the generic elongase complex, which includes dehydration and the final reduction, and the elongase process restarts with the novel 20 carbon dihydroxyacyl-CoA, probably catalysed by another FAE (Li *et al.*, 2018). The subsequent two rounds of elongations from 20 into 24 carbon, seem to follow the complete cycle of the generic elongase process in *O. violaeus* and results in nebraskanic FA, see figure 8. The discontinued elongation thus represents yet an additional way to introduce hydroxyl groups at novel positions compared to FAD2-like hydroxylases. The synthesis of wuhanic acid (7,18-OH-24:2$\Delta^{15,21}$) follow the same route as for nesbraskanic acid except that 12-OH-18:1Δ^9 is desaturated into densipolic acid, 12-OH-18:2$\Delta^{9,15}$, before elongation with the divergent KCS.

Transgenic Arabidopsis expressing both Ov*FAD2-2* and Ov*FAE1-1* produces nebraskanic FA while wuhanoic FA production requires incorporation on an additional elongase, OvFAE1-2. *O. violaceus* may also contain additional specialised enzymes facilitating the reductions and dehydration in the elongase complex (Li *et al.*, 2018).

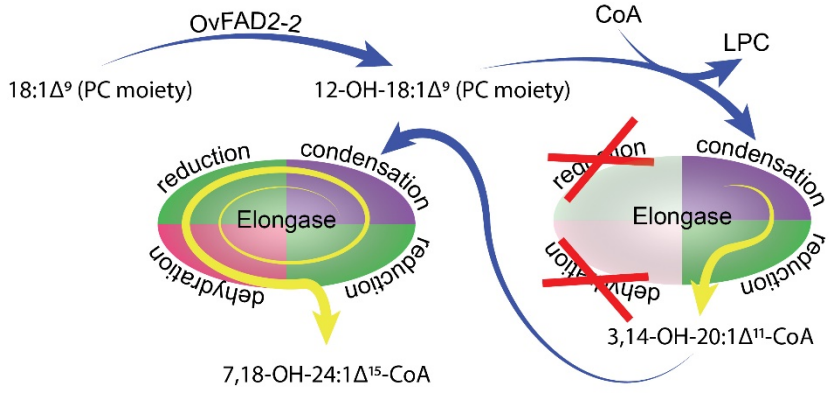

Figure 8 depicts a proposed biosynthetic pathway, resulting in nebraskanic acid in *O. violaceus*. OvFAD2-2 introduce a hydroxyl group at Δ^{12} on oleate acid associated with PC. A discontinued elongation cycle incorporates a second hydroxyl group at Δ^{3}. Two additional complete elongation cycles give rise to nebraskanic acid. The individual steps of the elongation cycle resemble that of fatty acid synthase where CoA substrates are used instead of ACP substrates, see figure 2. ***Abbreviations***: **FAD2 -** Fatty acid desaturase 2, **CoA** – coenzyme A, **LPC** – lysophosphatidylcholine, **PC** – phosphatidylcholine

The diversity of UFAs in plants is vast, and even though scientists have elucidated how several plants can modify them into their final configuration, the major part of their biosynthesis are yet unresolved. Researchers have also made significant progress on, albeit not yet wholly clarified, how plants can channel these UFAs into TAG through acyltransferases with pronounced specificities. Plants producing high levels of UFAs have often developed mechanisms excluding UFAs in membrane lipids, probably to avoid potentially detrimental effects in the membranes (Millar *et al.*, 2000). The evolutionary advantage of preserving a significant proportion of UFAs in the seed oil, or derivatives of these, might come from their novel properties. Some seed oils high in UFAs are known to be laxative, emetic and poisonous and may thus prevent herbivores from consuming the plant. Other UFAs are a suitable precursor for substances with protective effects against pathogens.

2.6 Engineered seed oil quality through conventional plant breeding.

B. napus, is a dual-purpose crop where varieties belonging to oilseed rape contains high levels of erucic acid whereas canola varieties are virtually free from erucic acid. Breeders developed canola or low erucic acid rapeseed (LEAR), in response to raised concerns about potentially detrimental health issues of consuming erucic acid (Beare *et al.*, 1957). The developing seed embryo of *B. napus* constitutes a substantial part of the *B. napus* seed, and it is the embryonic genotype, not the maternal, that determines the oil quality in *B. napus* (Downey & Harvey, 1963). The erucic acid content in *B. napus* seed oil is to a great extent determined by two loci, E1 and E2, which have a quantitative additive effect (Harvey & Downey, 1964). Breeders developed lines with two Mendelian recessive alleles, e1 and e2, which decreases the erucic acid content drastically. Two FAE1 genes, *FAE1.1* and *FAE1.2*, are colocalised with E1 and E2, respectively. Both *FAE1.1* and *FAE1.2* encodes KCS enzymes catalysing the rate-limiting condensation in the elongase complex (Fourmann *et al.*, 1998). *B. napus* probably arose in a spontaneous cross between *Brassica rapa* L. and *Brassica oleracea* L. and *B. napus* is thus an amphidiploid. The amino acid sequence of FAE1.1 is similar to that of FAE1 from *B. rapa* and FAE1.2 to that of *B. oleracea* (Fourmann *et al.*, 1998). The Bn*FAE1.2* allele from canola contains a frameshift leading to an incomplete protein due to a premature stop codon (Fourmann *et al.*, 1998). Comparison of FAE1.1 amino acid sequences from the rapeseed cultivar Askari and the canola variety Dakkar reveal that a single amino acid substitution renders the FAE1.1 enzyme of the canola FAE1 inactive (Han *et al.*, 2001). Continuous improvement of canola through breeders' efforts during almost 60 years development has led to an oil crop with seeds free from erucic acid and with a low glucosinolate content.

Breeders have, in parallel to the development of canola, intentionally increased the erucic acid content in the seed oil of high erucic acid rapeseed (HEAR). The increased demand for erucic acid and its derivates has intensified the development of HEAR varieties. Traditional *Brassica* oilseed crops produce seed oils with approximately 45 mol % of erucic acid. Conventional breeding efforts have increased the erucic acid content up to 50-55 mol % in commercial varieties (McVetty & Scarth, 2002). Stereopositional distribution of erucoyl moieties in seed oil TAG within Brassicaceae reveals that erucic acid is in most cases virtually absent on the *sn*-2 position (Taylor *et al.*, 1994). LPAT acylates the *sn*-2 position of LPA into PA in the Kennedy pathway, see figure 5. Determination of *B. napus* LPAT substrate specificity strongly indicate that it is incapable of using 22:1Δ^{13}-CoA as acyl donor. The inability of *B. napus* LPAT

to acylate the *sn*-2 position with erucic acid efficiently restricts the theoretical maximum concentration of erucic acid in TAG to 2/3 (Cao *et al.*, 1990).

The inherent theoretical maximum concentration of erucic acid in *B. napus* is challenging to circumvent with conventional breeding without breeding material containing a suitable LPAT. Breeders and researchers have thus resorted to modern biotechnological approaches instead of trying to further increase the erucic acid content by conventional breeding.

2.7 Engineered seed oil quality through a biotechnological approach

Genetic engineering provides researchers and breeders with tools and opportunities that enable the production of novel oil qualities in oil crops. Many of these oil qualities are impossible or extremely time-consuming to achieve with conventional breeding.

Researchers have screened the substrate specificity of LPATs from several species with a high content of VLCFA to identify candidate genes capable of circumventing the inherent inability of *B. napus* to acylate the *sn*-2 position of LPA with erucic acid. Two LPATs from species within the genus *Limnanthes*, *L. alba* and *L. douglasii*, LdLPAT and LaLPAT respectively, have the catalytic capability to facilitate the incorporation of erucic acid at the *sn*-2 position of LPA (Löhden & Frentzen, 1992; Cao *et al.*, 1990). Species within the genus *Limnanthes* produce seed oils with a TAG composition comprised almost exclusively of VLCFA, far beyond the 2/3 limit of *B. napus*. Δ^5-eicosenoic acid ($20{:}1\Delta^5$) comprises the majority of the VLCFA in the seed oil in *Limnanthes* species, but the two LPATs accepts erucoyl-CoA nonetheless (Miller et al., 1964). Transgenic *B. napus* expressing the Ld*LPAT* or La*LPAT* in seeds during development does not gain an increase erucic acid content compared to the wildtype (Weier *et al.*, 1997; Brough *et al.*, 1996; Lassner *et al.*, 1995). The erucic acid is, however, more evenly distributed at all three *sn* positions (Brough *et al.*, 1996; Lassner *et al.*, 1995). Suspicions that the elongase complex in *B. napus* is unable to provide sufficient amounts of erucic acid to the acyltransferases in these transgenic plants led scientists to implement a more elaborate approach.

The elongase complex in *B. napus* utilises $18{:}1\Delta^9$ for elongation. As do FAD2 and indirectly FAD3 for desaturation during the synthesis of $18{:}2\Delta^{9,12}$ and $18{:}3\Delta^{9,12,15}$. The concurrent elongation and desaturation processes thus compete

for the same substrate, 18:1Δ^9. Scientists have cloned *FAE1.1* and *FAE1.2* from a HEAR cultivar and co-expressed them with Ld*LPAT* in a breeding line named 'HELP'. Some of these transgenic plants managed to produce seed oils with up to 72 % erucic acid in greenhouse trials. On average, there was an increase of 9 % of erucic acid in the transgenic plants compared to the breeding line 'HELP' (Nath *et al.*, 2009).

'HELP is an acronym for 'High Erucic Low Polyunsaturated', and the seed oil of this breeding line contains approximately 50 % erucic acid and 7 % polyunsaturated FAs (PUFA), predominantly 18:2$\Delta^{9,12}$ and 18:3$\Delta^{9,12,15}$. Another transgenic *B. napus* produce a seed oil which has a fatty acid composition that resembles that of 'HELP'. The seed-specific downregulation of *FAD2* in these transgenic plant decreases the amount of 18:2$\Delta^{9,12}$ and 18:3$\Delta^{9,12,15}$ in the seed oil to less than 5% compared to approximately 20 % in the wildtype *B. napus*. The erucic acid content increases at the same time, from 42 % to 45 % (Shi *et al.*, 2017). While the seed-specific downregulation of *FAD2* through an RNAi construct significantly affect the seed oil composition, it does so without other severe phenotypic changes of the plant (Shi *et al.*, 2017).

Crambe is an alternative oil crop suitable for the production of erucic acid. Crambe has several benefits compared to HEAR. Both the seeds and the seedpods are distinctly different from that of the cultivated *Brassica* ssp., reducing the risk of misidentification between a seed lot intended for food and high erucic containing material. Further, crambe does not seem to hybridise with cultivated *Brassica* crops, which is an advantage compared to HEAR (FitzJohn *et al.*, 2007). Cultivation of HEAR inevitably includes risks of erucic acid contamination of canola. HEAR cultivated in the vicinity of canola, for example, can facilitate cross-pollination (Warner & Lewis, 2019). The embryonic genotype determines the FAs composition in *B. napus* and the absence of erucic acid in canola relies, at least partially, on Mendelian recessive alleles. No such consideration must be taken into account while producing erucic acid in crambe as it does not cross-pollinate with cultivated *Brassica* spp. Crambe is currently already regarded as a source for industrial feedstock rather than food, and the erucic acid content in the seed oil is comparable to that of HEAR (Lazzeri *et al.*, 1997).

An attempt to increase the erucic acid content in a crambe cultivar ´Galactica´ has sofar been modestly successful. The *sn*-2 position in ´Galactica´ seed oil TAG is, as in *B. napus*, very seldom acylated with an erucoyl moiety. The total erucic acid content is, however, almost 60 %, and the amount of PUFA

constitutes 14 % (Li *et al.*, 2012). Li *et al.* (2012) implemented an approach based on the gained experience of transgenic HEAR; including a down-regulation of Ca*FAD2* through an RNAi in concert with the overexpression of Bn*FAE1* and Ld*LPAT*. Analysis of the seed oil from the transgenic crambe reveal a significant increase in erucic acid content, up to 73 %, similar to the best-performing transgenic HEAR lines (Li *et al.*, 2012; Nath *et al.*, 2009). A more even distribution of erucic acid between the three *sn* positions is apparent in the seed oil, albeit low amounts of trierucin (Li *et al.*, 2012). There is, however, no net gain of erucic acid in the transgenic crambe; due to a yield penalty on an oil per seed basis (Ortiz *et al.*, 2020). The erucic acid content in the DAG pool is much higher in developing seeds of the transgenic lines compared to the cultivar 'Galactica' throughout the seed development. Further, the difference in erucic acid content between 'Galactica' and the transgenic lines is more articulated in DAG than it is in TAG during seed development (Guan *et al.*, 2014).

The highest level of erucic acid content recorded is from, a perhaps unexpected plant, *Tropaeolum majus* L., garden nasturtium (Ohlrogge *et al.*, 2018; Taylor *et al.*, 2010). *T. majus* has gained much attention due to the seed oil that contains almost 80 % erucic acid. The seeds of *T. majus* are however not very suitable for oil production as they contain only ~10 % oil. Studies of three identified genes from *T. majus* involved in lipid metabolism, Tm*LPAT*, Tm*DGAT1* and Tm*FAE* indicate that these are potential candidates for increasing the erucic acid content in transgenic crops (Taylor *et al.*, 2010; Xu *et al.*, 2008; Mietkiewska *et al.*, 2004). Expression of Tm*DGAT1* in a HEAR breeding line increases the oil content by 3-8 %, but there is, unfortunately, no significant increase of erucic acid content (Xu *et al.*, 2008).

Another area of interest where scientists have identified opportunities for improvement with engineered oil qualities is for feed in aquaculture and the production of long-chain ω-3 PUFA (LC-PUFA) in oil crops. ω-3 in this context refers to a group of long-chain PUFAs where the last double bond is situated three carbons from the ω carbon. One of the outcomes during the United Nations world summit on sustainable development in 2002 regarding fisheries was to:

> 'Maintain or restore stocks to levels that can produce the maximum sustainable yield (MSY) with the aim of achieving these goals for depleted stocks on an urgent basis and where possible not later than 2015' (Ye *et al.*, 2013).

Nevertheless, assessments estimate that more than 2/3 of monitored fish populations accounting for 80 % of the global fish populations in 2008 were overharvested (Ye *et al.*, 2013). The annual catch by global fisheries is stable

since the 1990s and yields approximately 90 million tonnes of fish per year. There is a continuously increased demand for fish due to a growing human population, and approximately 16 % of the total animal-protein intake of humans are of marine origin. The yield of fish in aquaculture has already increased tenfold from 1980 to 2010. Predictions estimate that the yields of aquacultured fish will exceed that of the global fisheries by 2030 as a response to meet human's demand for more fish (Kobayashi *et al.*, 2015).

Several studies advocate dietary intake of oily fish, due to the beneficial effects of LC-PUFAs, especially docosahexaenoic acid (DHA, $22{:}6\Delta^{4,7,10,13,16,19}$), and eicosapentaenoic acid (EPA, $20{:}5\Delta^{5,8,11,14,17}$) (Harris, 2004). Most animals, including fish species, are unable to *de novo* synthesise PUFAs, with a few exceptions within invertebrates. PUFAs are, therefore, an essential part of the dietary intake for most animals (Malcicka *et al.*, 2018). Successful aquaculture, therefore, relies on PUFA supplemented feed. The LC-PUFAs in wild fish originates from single-celled marine organisms that fish eat (Harris, 2004). Salmonid fishes are predatory and popular within aquaculture. Salmonids can, to some extent, synthesise both EPA and DHA from plant seed oils containing 18 carbon PUFAs (Tocher, 2015). Fish fed with PUFA containing plant oils are, however, not able to maintain the high levels of LC-PUFA as fish fed with fish oil or wild living relatives (Tocher, 2015). Fish feed for aquacultures, therefore, contains fish oils derived from wild fish populations. The price of these commodities has recently increased drastically, and predictions indicate that they will continue to increase (Kobayashi *et al.*, 2015). Feeding aquaculture with marine-based feed products also counteracts one of the benefits with aquaculture as it endangers the marine populations through potential overharvesting.

Scientists have, for the last two decades, investigated the possibility to produce LC-PUFA in oil crops to meet the increasing demand (Abbadi *et al.*, 2004; Qi *et al.*, 2004). Small aquatic organisms, e.g. microalgae, bacteria and diatoms can synthesise LC-PUFA via two distinct pathways, the aerobic and the anaerobic. The anaerobic pathway occurs predominantly in bacteria, which produces LC-PUFA cyclically from malonyl-CoA and acetyl-CoA. PUFA synthase is a multi-enzyme complex that catalyses the two-carbon elongations and desaturations in the anaerobic pathway. Amino acid alignment of PUFA synthase domains reveals that PUFA synthase represents a hybrid between polyketide synthases (PKS) and FAS (Metz *et al.*, 2009). The aerobic pathway is more similar to the FAs modification and synthesis that occurs in oil crops than the anaerobic (Napier *et al.*, 2015). Several oil crops already produce

significant amounts of precursors to the aerobic pathway. In the aerobic pathway, consecutive desaturation and elongation of $18{:}2\Delta^{9,12}$ results in DHA and EPA, see figure 9. Two intertwined routes, ω-3 and ω-6, exists within the aerobic route. The ω-3 route relies on $18{:}3\Delta^{9,12,15}$ while the ω-6 route uses $18{:}2\Delta^{9,12}$. The aerobic pathway also contains additional alternative routes. Desaturation of 18 carbon substrates by Δ^6 desaturases and subsequent elongation predominates over the alternative route where elongation occurs before desaturation of a Δ^8 desaturase, see figure 9 (Napier *et al.*, 2015).

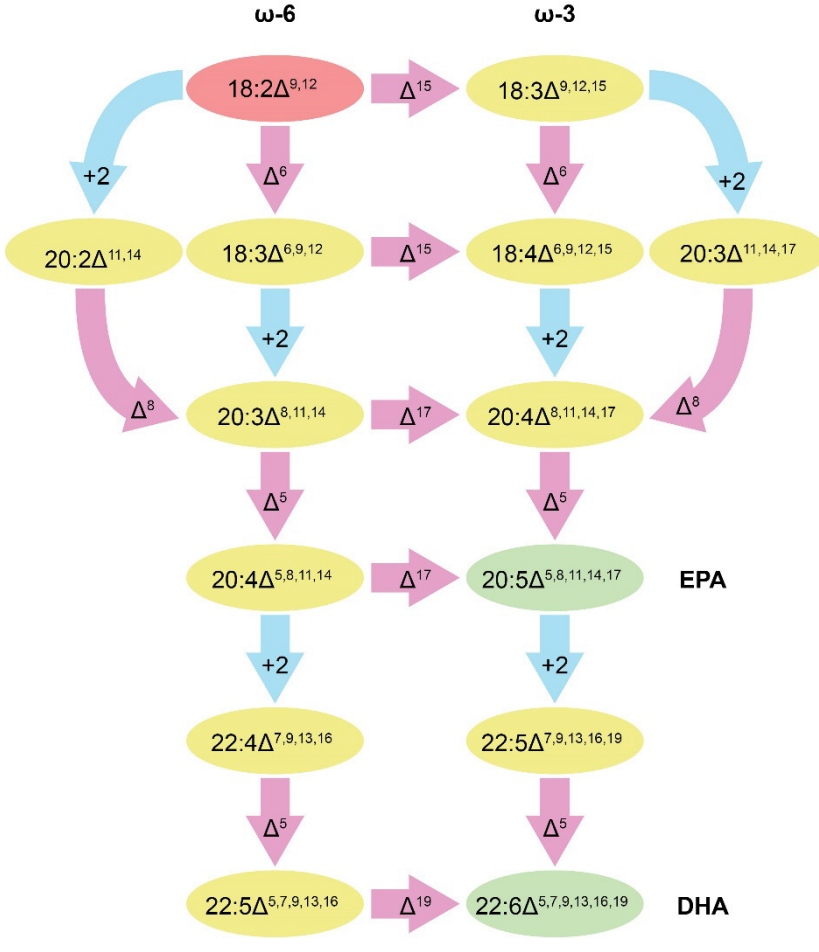

Figure 9 illustrates several possibilities for the synthesis of both EPA and DHA. Multiple alternating desaturations (pink) and elongations (blue) of the plant available $18{:}2\Delta^{9,12}$ and $18{:}3\Delta^{9,12,15}$ mimics the aerobic pathway of many microalgae (Napier *et al.*, 2015; Petrie & Singh, 2011). ***Abbreviations***: **DHA** - docosahexaenoic acid, **EPA** - eicosapentaenoic acid

Scientists have identified all the genes coding for the enzymes responsible for the synthesis of LC-PUFA within both the aerobic and anaerobic pathway (Petrie & Singh, 2011). Extensive and laborious efforts have resulted in a successful production of LC-PUFA in transgenic oil crops. Dow AgroSciences LLC and DSM Nutritional Products LLC presented in 2016 canola synthesising LC-PUFA through the anaerobic pathway. Field-grown transgenic canola produces a seed oil that contains up to 4.4 % DHA and 0.6 % EPA (Walsh *et al.*, 2016).

Other research groups have successfully implemented the aerobic pathway in both canola and *Camelina sativa* (L.) Crantz (Napier *et al.*, 2019). The production of LC-PUFA through the aerobic pathway in oil crops is by no means a small feat; multiple components have to function coherently for efficient synthesis of LC-PUFA and subsequent incorporation into TAG, as seen in figs. 5 and 9. Two groups, BASF/Cargill and Nuseed/CSIRO/GRDC, have in parallel developed transgenic canola varieties using slightly different approaches, but with similar amounts, ~12 % LC-PUFA in the seed oil (Napier *et al.*, 2019). The two canola varieties differ however in the FA composition, one with high levels of EPA and the other with high levels of DHA. The two groups used rather large constructs, consisting of more than 32 kb, containing either seven or 11 genes, dedicated to LC-PUFA that originates from a wide range of organisms (Connelly & MacIntosh, 2018; Sottosanto *et al.*, 2018).

Transgenic *C. sativa* is also an up-and-coming candidate for producing LC-PUFA and can produce a seed oil containing very high levels of these FAs, exceeding the publically announced levels achieved in transgenic canola. The seed oil of transgenic *C. sativa* lines can, so far, contain up to 24 % EPA (Ruiz-Lopez *et al.*, 2014). The highest published amount of DHA produced in a *C. sativa* line is currently ~12 %, and this line contains ~4 % of EPA as well (Petrie *et al.*, 2014). A transgenic line of *C. sativa*, worth mentioning in this context is not producing the highest amounts of LC-PUFA, but the composition of both EPA and DHA, 11 % and 8 % respectively, and the overall of FA composition of the seed oil roughly resembles that of bulk fish oil (Ruiz-Lopez *et al.*, 2014).

The examples presented above illustrate that it is possible to engineer an oil quality with desirable traits using a biotechnological approach. It is not merely a vision with future applications. At present, we see several instances where transgenic oil crops already can replace harmful practices and replace less environmentally friendly alternatives. A robust understanding of plant lipid metabolism and the biochemical characterisation of involved enzymes underpins the future production of renewable oleochemical commodities.

3 Principles and justification of reoccurring methods

The papers included in this thesis make use of radiolabelled [^{14}C] substrates and microsomal preparations. The inherent nature of integral enzymes such as those in the Kennedy pathway precludes structural determination by protein X-ray crystallography. While biochemical *in vivo* studies can reveal essential fluxes and processes, parallel processes can make it virtually impossible to interpret the data for a single enzyme. In *B. napus*, for example, eight DGATs concurrently facilitate the acyl-CoA dependent formation of TAG, while PDAT synthesises TAG from PC and DAG simultaneously. To determine the substrate specificity of a single DGAT in such a complex environment is, to say the least very challenging, if not impossible.

Introduction, overexpression or downregulation of a gene in stable transformants can provide valuable information but is time-consuming. Transient gene expression of *Agrobacterium tumefaciens* Smith & Townsend in, for example, *Nicotiana benthamiana* Domin is another alternative but is dependent on the endogenously available substrates. Co-expression of additional transgenes facilitating the synthesis of the desired substrate may thus be necessary.

We base most of the investigations we carry out as described in this thesis on microsomal preparations derived from either developing plant embryos or yeast cultures. Carefully selected substrates and appropriate buffers enable detailed studies of integral proteins present in the microsomal membranes. Microsomal preparations consist of reconstituted vesicles derived from membranes of the ER. We recover the microsomal membranes through ultracentrifugation of homogenised cells after removal of larger cell debris. We frequently express integral plant-derived lipid metabolism involved genes in yeast. Yeast is capable

of producing TAG, and the four genes *ARE1*, *ARE2*, *DGA1* and *LRO1*, contribute to the synthesis of TAG. Disruption of these four genes results in yeast, still viable but devoid of TAG. We, therefore, often utilise such a TAG devoid yeast, yeast strain H1246 (Sandager *et al.*, 2002), to study lipid metabolism enzymes see, figure 10. The TAG produced by recombinant H1246 expressing, for example, a *DGAT* is thus exclusively produced by the transgene in question. Yeast cultures are also facile to transform, cultivate and maintain.

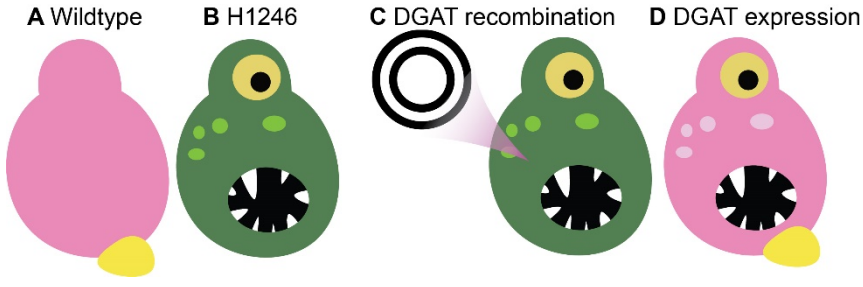

Figure 10 illustrates the principles behind expressing TAG producing acyltransferases in H1246. **A** Wildtype yeast is fully capable of producing TAG. **B** The disruptions of four genes in H1246 results in a viable yeast strain, H1246, devoid of TAG. **C** Introduction of a DGAT restores the capability to produce TAG, **D** once expressed. The generated TAG is hence the product of the introduced enzyme, enabling detailed biochemical characterisation of such acyltransferases

Most lipid substrates, both radiolabelled and unlabelled, are meticulously synthesised in our lab from FAs based on Sten Stymne lifelong experience within biochemistry. Many of these substrates are currently unavailable on the market or prohibitively expensive to procure. We produce acyl-CoA, various DAG-, PC-, LPC-, LPA- TAG-, and unfortunately, now and then, inadvertently MAG-species on a routine basis. These substrates are an absolute necessity for the methods we apply to characterise the enzymes involved in lipid metabolism.

Our assays consist of defined amounts of necessary substrates and microsomal preparations diluted in an appropriate buffer. We incubate the assays during a defined period and the combination of microsomal protein amount, time and substrate are usually adjusted to ensure close to linear conditions during the assays. After we have performed the assays, we analyse the lipids (extracted into chloroform), both remaining, degraded and produced during the incubation. We use either thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) to separate the products in parallel with a liquid

scintillation counter (LCS), which determines the total radioactivity in an aliquot of the chloroform extract. A Packard InstantImager Electronic Autoradiography system determines the relative contribution of the total radioactivity as determined by LCS from each lipid class separated by TLC. The radioactivity detector Raytest Ramona Star determines the relative contribution of lipid classes in samples separated by HPCL.

We have previously generally used [^{14}C] di-6:0-DAG as acyl-acceptor during characterisation of DGAT enzymes, due to the water-solubility of this DAG species. Longer acyl-chain-DAG species such as di-18:1Δ^9-DAG does not readily dissolve in buffers and is thus challenging to add to the microsomal preparation in a way that makes them accessible for the enzymes. We have recently developed a robust method to conduct assays with various long-chain-DAG species, which uses DAG species dissolved in a small volume of DMSO.

4 Results and insights derived from the papers

Results at a glance

Paper I
The DGAT2 enzymes from *B. napus* with similar amino acid sequences fall into two categories based on their drastically different acyl-CoA specificities when assessed with di-6:0-DAG.

Paper II
The acyl-CoA specificity of DGAT2 enzymes is significantly affected by a specific region residing in the first third of the amino acid sequence. The identified segment contains two predicted transmembrane helices which are abundant in a wide range of plant-derived DGAT2 enzymes.

Paper III
Several DGAT genes, of both DGAT1 and DGAT2 present in the crambe genome, produces enzymes that lack or exhibit severely reduced capacity to use di-22:1Δ^{13}-DAG as a substrate. The reduced ability to acylate di-22:1Δ^{13}-DAG by these DGAT enzymes may pose a significant drawback in the pursuit to achieve a seed oil with a very high content of erucic acid in crambe.

Paper IV
We elucidate the elusive DGAT2 sequence of *T. majus*, which is a potential candidate for circumventing the bottleneck identified in paper III. Biochemical characterisation of both TmDGAT1 and TmDGAT2 reveal that these too, just as the crambe DGAT enzymes, are either unable or possess a reduced capacity to acylate di-22:1Δ^{13}-DAG.

Paper V
Here we characterise several essential enzymes involved in lipid metabolism from *C. sativa*. The characterised DGAT2 exhibit a similar specificity as one of the DGAT2 categories of *B. napus* assessed in paper I. Further, the *C. sativa* PDCT is unable to use ricinoyl containing DAG species. The PDCT is not only unable to use ricinoyl containing DAG species, but the presence of ricinoleoyl containing DAG species also seem to inhibit head group interchange by PDCT of other DAG species into PC as well.

Careful considerations regarding the compatibility of the intrinsic lipid synthesis are essential when introducing the synthesis of exogenous FAs or in other ways re-engineering the TAG FA composition in an oil crop. Several factors within the intrinsic lipid synthesis will affect the outcome. The modification of FAs into UFAs often require that a specific FA is associated with different lipids throughout its modification to its final, desired configuration. For example, many, but not all desaturases, utilise FA-moieties of PC as substrates while the extraplastidic elongase complexes in plants utilise acyl-CoA as a substrate which requires an efficient interchange of FAs between PC and acyl-CoA. The substrate specificity of the endogenous enzymes involved in lipid metabolism will thus inevitably affect the result. Concurrent modifications of FAs and lipid assembly may also compete for the initial substrate or its intermediates. Spatial and temporal variations of lipid species occur within developing seeds of several species, as is the case with seeds of, e.g. *B. napus* and *C. sativa* (Marmon *et al.*, 2017; Usher *et al.*, 2017; Woodfield *et al.*, 2017). Further, the enzymatic capacity of acyltransferases within microsomal preparations from developing seeds is known to vary drastically between different developmental stages (Furmanek *et al.*, 2014).

All of these aspects, and several additional, affects the final FA composition in the final seed oil. Adjustments that counteract any potential incompatibility between the endogenous lipid metabolism and the desired products may very well add to the complexity of an approach to engineer the seed oil composition. Rigorous biochemical characterisation of the lipid metabolism within a plant provides vital pieces of information, aiding the design process of how to alter the FA composition of a seed oil successfully.

The main focus in this thesis is on acyltransferases, DGATs in particular, and especially the DGAT2 forms. DGAT is a significant contributor to the accumulating seed oil in many plants. The acyl-donor specificity directly affects the FAs composition at the *sn*-3 position of TAG. The acyl acceptor specificity of DGAT, however, may act as a filter and exerting a distinct selection of DAG species to be acylated. Thus, DGAT specificity may affect the FAs composition on all three *sn* positions in TAG. Several plants producing UFAs contains DGAT2 enzymes that possess specificities prone to prefer or at least readily accept the abundant UFA. The systematic biochemical characterisation of DGAT enzymes aids the fundamental understanding of lipid metabolism. Further, an increasing number of characterised enzymes enables the recognition of patterns of motifs related to the specificity emerging within the DGAT2 amino acid sequences.

In **paper I**, we provide a robust characterisation of several DGAT enzymes derived from *B. napus*. The *B. napus* genome as an amphidiploid contains four genes for each DGAT. We identified four *DGAT1* alleles and five *DGAT2* alleles during a screening of DGAT genes present in the genome of the canola variety 'Monolit' at the HEAR variety 'Maplus'. Two of the *DGAT2* alleles representing *Bna*A.DGAT2.c differed slightly on the amino acid level between the two cultivars but behaved similarly in later acyl-CoA specificity determinations. We assessed the acyl-CoA specificity for each of the enzymes encoded by the identified alleles with the artificial acyl acceptor di-6:0-DAG in recombinant yeast. The four DGAT1, all exhibited a similar acyl-CoA specificity while the DGAT2 specificities differentiate. The DGAT2 enzymes fall into two categories based on their acyl-donor specificities, those that primarily accept $18{:}3\Delta^{9,12,15}$-CoA and those that accept $22{:}1\Delta^{13}$-CoA at similar rates as $18{:}3\Delta^{9,12,15}$-CoA, see figure 11A and B. These two groups of DGAT2 enzymes that differ starkly in the acyl-CoA specificity have however very similar amino acid sequences. Phylogenetic analyses indicate that both ancestral parent of *B. napus*, *B. rapa* and *B. oleracea*, provided one of each type of the DGAT2 categories, see fig 11C. All *DGAT2* genes are, however, annotated on chromosomes from the A genome, derived from *B. rapa*.

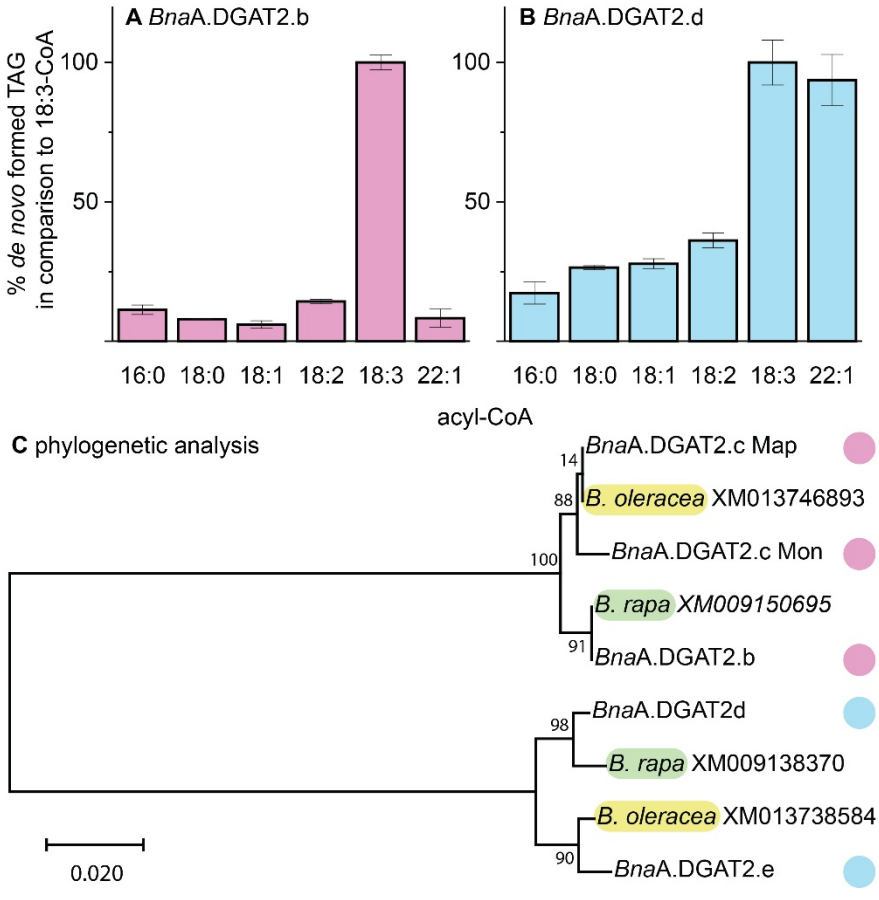

Figure 11 **A** the acyl-CoA specificity of *Bna*A.DGAT2.b is representing the category *of B. napus* DGAT enzymes that prefer 18:3-CoA. **B** The acyl donor specificity of *Bna*A.DGAT2.d represents the category of *B. napus* DGAT enzymes that prefers both 18:3$\Delta^{9,12,15}$-CoA and 22:1Δ^{13}-CoA at a similar level. We assessed both DGAT2 enzymes activities with di-6:0-DAG as acyl donor under close to linear conditions. Error bars denote standard deviation, n= 3. **C** A phylogenetic analysis of the DGAT amino acid sequences of *B. napus* and its ancestral parents DGAT sequences is based on the maximum likelihood method algorithm in mega X with 1000 bootstraps. Pink dot indicates that a DGAT belonging to the DGAT2 category presented in **A** while blue dot indicates that a DGAT2 belongs to the category represented in **B**

The DGAT2 enzymes of *B. napus* provide an excellent opportunity to study motifs/amino acid segments that influence the acyl-CoA specificity of DGAT2 enzymes as they differ in their acyl-CoA specificity despite their similar amino acid sequences. **Paper II** describes the approach we implemented to elucidate the potential presence of a confined region of the amino acid sequence of DGAT2 that affect the acyl donor specificity. The iterative approach includes the design of chimeric enzymes and the screening process of the chimeric enzymes for an altered acyl donor specificity. The characterisation of the first chimeric enzymes, where large segments of amino acids from a 22:1Δ^{13}-CoA accepting enzyme substitutes the corresponding sequence of an enzyme predominantly accepting 18:3$\Delta^{9,12,15}$-CoA enables a rough estimation of where regions of interest for substrate specificity are situated. The screening, conducted on microsomal preparations of recombinant yeast include separate assays with [^{14}C]-labelled di-6:0-DAG and either 18:3$\Delta^{9,12,15}$-CoA or 22:1Δ^{13}-CoA. A shift in the proportion of *de novo* formed TAG during assays between 18:3$\Delta^{9,12,15}$-CoA and 22:1Δ^{13}-CoA compared to the parental 18:3$\Delta^{9,12,15}$-CoA preferring enzyme, indicate a change in acyl-donor specificity, see figure 12A and B. New generations of chimeric enzymes with a design based on the results from the screening process of the previous generation of enzymes allowed us to decrease the size of the amino acid substitution gradually. We managed to identify a region that significantly affects the acyl donor specificity while implementing this strategy, see figures 12A-E and 13.

The identified region, situated in the first third of the DGAT2 enzymes contains two predicted transmembrane helices. Predictions indicate that these two transmembrane regions reoccur in a wide range of plant-derived DGAT2 enzymes. We designed a new chimeric enzyme to elucidate whether the importance of this region confines to *B. napus* DGAT2 enzymes or whether it may be applicable in other species as well. The new chimeric enzyme contains a backbone of Arabidopsis DGAT2 substituted in the identified region by a 22:1Δ^{13}-CoA accepting *B. napus* DGAT2. The acyl-CoA specificity of the chimeric At*Bna*DGAT2 differs significantly from that of the wildtype Arabidopsis DGAT2, and it exhibits a drastic reduction in the preference for 18:3$\Delta^{9,12,15}$-CoA, down to a level comparable to 22:1Δ^{13}-CoA, see figure 12D and E. This change in specificity indicates that the identified region may not only be of importance in *B. napus* but also for other plant-derived DGAT2 enzymes.

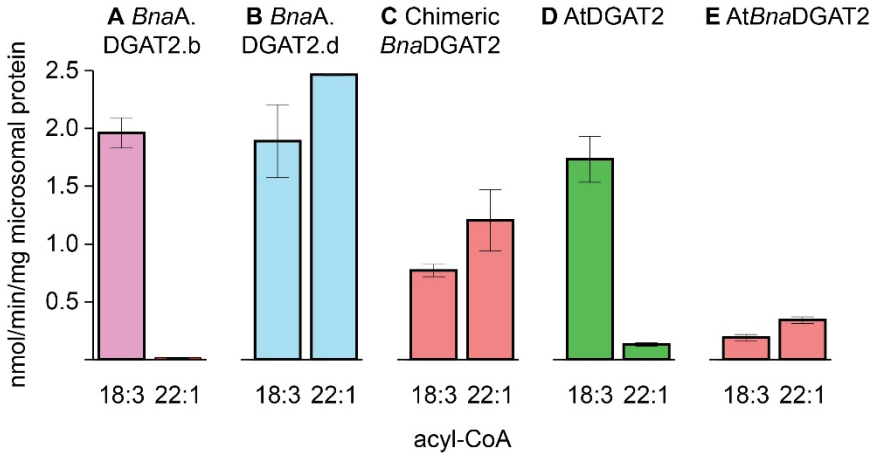

Figure 12 Specificity towards 18:3$\Delta^{9,12,15}$-CoA and 22:1Δ^{13}-CoA with supplementing [^{14}C]-di-6:0-DAG of parental wildtype enzymes from *B. napus* (**A** and **B**) and Arabidopsis (**D**) and chimeric enzymes (**C** and **E**) **A** *B. napus* parental wildtype DAGT2 form that prefers 18:3$\Delta^{9,12,15}$-CoA predominantly. **B** *B. napus* parental wildtype form preferring both 18:3$\Delta^{9,12,15}$-CoA and 22:1Δ^{13}-CoA at a similar rate. **C** chimeric *B. napus* enzyme where B substitute the identified delimited region in the A enzyme. **D** Arabidopsis wildtype parental enzyme. **E** A chimaera where a region of the *B. napus* enzyme B replace the region of interest in the Arabidopsis DGAT2 amino acid sequence. n=3, error bars denote the standard deviation

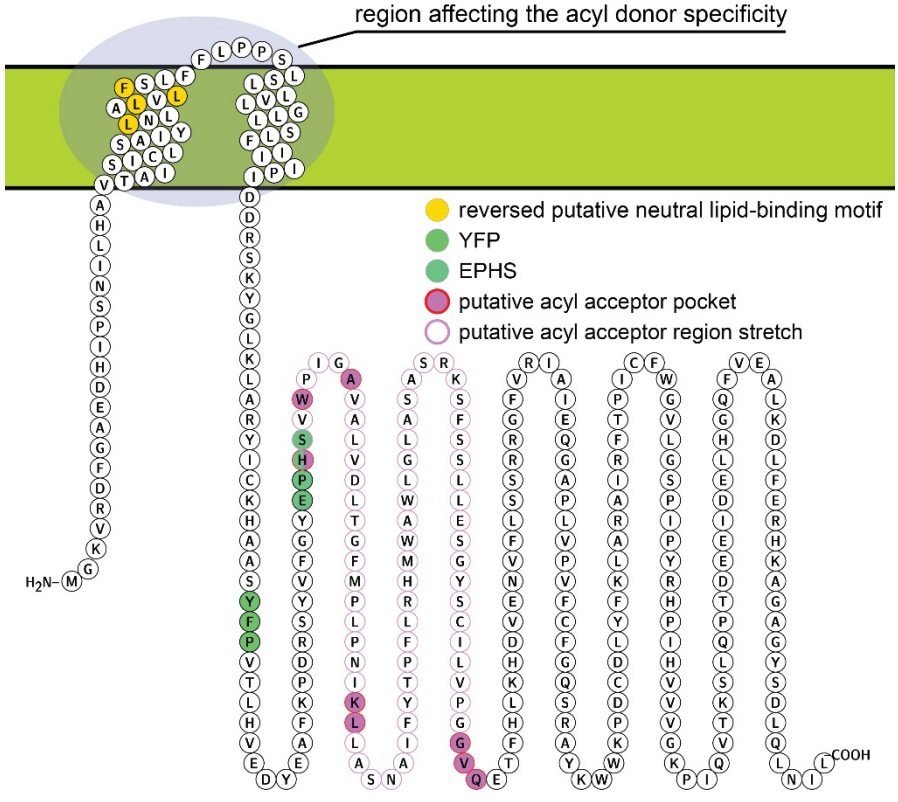

Figure 13 depicts the amino acid sequence of *Bna*A.DGAT2.d, predicted transmembrane helices, previously described motifs and the region affecting the acyl donor specificity, identified in **paper II** (semitransparent blue ellipse). A putative neutral lipid-binding motif (yellow) is present, however in reversed order compared to Stone *et al.* (2006). YFP and EPHS (green) are both essential for DGAT catalytic activity (Liu *et al.*, 2011). Highlighted pink residues represent a putative acyl acceptor pocket (Lu *et al.*, 2020; Turnbull *et al.*, 2001).

The third paper, **paper III**, focuses on crambe and a potential bottleneck posed by DGAT substrate specificities. A previous investigation elucidating potential bottlenecks of erucic acid accumulation in transgenic high erucic crambe reveal that developing seeds possess a much higher accumulation of di-22:1Δ^{13}-DAG compared to the increase of erucic acid in TAG. A hypothesis arose that the intrinsic acyltransferases, primarily DGAT, that catalyse the final step in the synthesis of TAG are unable to utilise di-22:1Δ^{13}-DAG as a substrate efficiently. We successfully identified and cloned eight alleles encoding *DGAT* in crambe, four of each *DGAT1* and *DGAT2*. Acyl-CoA substrate specificity determined when using di-6:0-DAG revealed that 22:1Δ^{13}-CoA was one of the most favoured of all the acyl-CoA tested by all enzymes but one. The latter enzyme, a DGAT2, deviate and strongly prefers 18:3$\Delta^{9,12,15}$-CoA over 22:1Δ^{13}-CoA. The deviating DGAT2 enzyme differs not only in the specificity of acyl donors but also in acyl acceptor preference.

We conducted a more detailed study of substrate specificities of one DGAT1 and one DGAT2, which both prefers 22:1-CoA as a substrate in combination with di-6:0-DAG. These more detailed studies include the use of DAG species likely to occur in developing seeds of crambe unlike the artificial di-6:0-DAG. The most frequently occurring DAG species utilised for TAG synthesis in wildtype crambe is *sn*-1-22:1Δ^{13}-*sn*-2-18:1Δ^{9}-DAG. Both of the tested DGAT

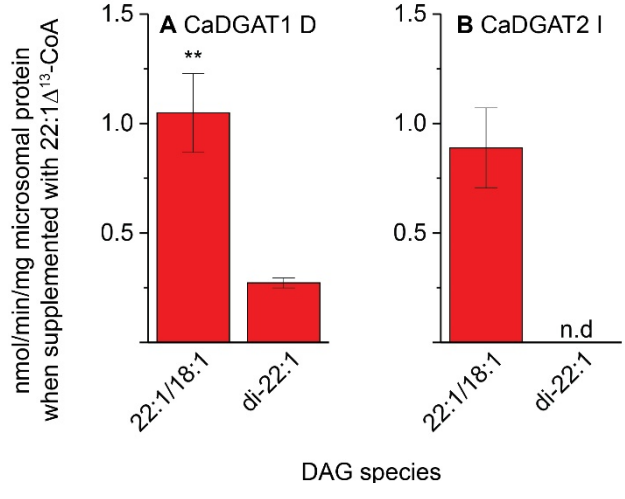

Figure 14 substrate specificities of crambe DGAT enzymes towards 22:1Δ^{13}-CoA in combination with the frequently occurring *sn*-1-22:1Δ^{13}-*sn*-2-18:1Δ^{9}-DAG or di-22:1Δ^{13}-DAG. **A** CaDGAT1 D **B** CaDGAT2 I, n=3 error bars denote standard deviation, significance determined by one-way ANOVA with posthoc Tukey's HSD test

enzymes readily accept *sn*-1-22:1Δ^{13}-*sn*-2-18:1Δ^{9}-DAG as acyl acceptor and can acylate the *sn*-3 position of this DAG with 22:1Δ^{13}-CoA, see figure 14. The situation is quite different with di-22:1Δ^{13}-DAG as acyl acceptor. The DGAT2 is unable to utilise the di-erucic acyl-acceptor while the DGAT1 is capable of catalysing the formation of TAG from di-22:1Δ^{13}-DAG but at a much-reduced rate compared to *sn*-1-22:1Δ^{13}-*sn*-2-18:1Δ^{9}-DAG, see figure 14. We also conducted similar long-chain DAG assays with the deviating DGAT2 enzyme as well. This enzyme was unable to use both *sn*-1-22:1Δ^{13}-*sn*-2-18:1Δ^{9}-DAG and di-22:1Δ^{13}-DAG. Thus, a substantial part of the endogenous TAG catalysing enzymes in crambe are unable to or at least very poor at, producing trierucin despite the presence of di-22:1-DAG in the transgenic crambe (Guan *et al.*, 2014).

The poor capability of the crambe DGAT enzymes to produce trierucin encouraged us to identify a suitable *DGAT* gene candidate to aid the enrichment of erucic acid in transgenic crambe. The identification of *DGAT2* from *T. majus*, with its record-high levels of erucic acid in the seed oil, has remained elusive despite repeated attempts to identify it. We re-evaluated publically available transcriptomic data in **paper IV** and found a partial nucleotide sequence potentially belonging to a DGAT2 enzyme. We retrieved a full-length sequence through a 5′/3′ RACE conducted on cDNA derived from developing seeds of *T. majus* with primers based on the partial sequence from the transcriptome.
The identified DGAT2 enzyme exhibit an extreme specificity towards

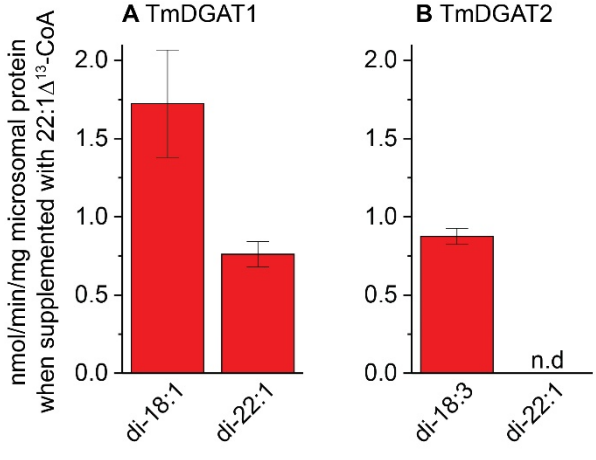

Figure 15 substrate specificities of *T. majus* DGAT enzymes towards 22:1Δ^{13}-CoA in combination with most preferred DAG species or di-22:1Δ^{13}-DAG. **A** TmDGAT1 **B** TmDGAT2, n=3 error bars denote standard deviation

18:3$^{\Delta 9,12,15}$-CoA when supplemented with di-6:0-DAG, which is more than sixfold larger than the second most favoured acyl donor. We assessed the acyl-CoA specificity of both TmDGAT1 and the newly identified TmDGAT2 with di-22:1Δ^{13}-DAG and again, as in crambe, TmDGAT2 is unable to utilise di-22:1Δ^{13}-DAG, while the TmDGAT1 enzyme can, see figures 14 and 15.

Transgenic *C. sativa* is, as demonstrated in the production of EPA and DHA, very suitable for the production of novel oil qualities. As seen in figure 9, synthesis of, e.g. EPA and DHA in an oil crop requires a wide range of introduced genes, where sequential alternating desaturations and elongations modify 18:2$\Delta^{9,12}$ and 18:3$\Delta^{9,12,15}$ into the final product (Connelly & MacIntosh, 2018; Sottosanto *et al.*, 2018). The characterisation of enzymes involved in lipid metabolism in *C. sativa* is still very valuable, despite the successful production of EPA and DHA as the incorporation of other FAs may be less compatible with the endogenous lipid metabolism. We, therefore, characterised several enzymes from *C. sativa* involved in lipid metabolism in **paper V**. Assessment of the acyl-donor specificity of CsDGAT1 and CsDGAT2 while providing di-6:0-DAG indicate that the CsDGAT1 exhibit very broad and indiscriminate specificity and readily accepts all assessed acyl-CoA substrates. CsDGAT2, on the other hand, express a specificity profile that resembles that of the *B. napus Bna*A.DGAT2.b, where 18:3$\Delta^{9,12,15}$ is highly preferred compared to the other tested acyl-CoAs, see figures 16A and 11A. Phylogenetic analyses also indicate a closer kinship of CsDGAT2 to the *Bna*A.DGAT2 enzymes with similar specificity profiles rather than the two *Bna*A.DGAT2 enzymes that readily accept 22:1Δ^{13}-CoA as substrate, see figure 16B. Characterisation of the *C. sativa* PDAT enzyme reveals that acyl donor specificity towards PC species is dependant of the acyl acceptor DAG species and changes significantly, just as with the DGAT enzymes in crambe.

Further, we investigated the *modus operandi* of the CsPDCT enzyme, catalysing the interconversion between DAG and PC. CsPDCT is unable to utilise DAG-species containing ricinoleoyl. Not only is CsPDCT unable to utilise these DAG species, but they also seem to inhibit the PDCT catalysed interconversion of other DAG species devoid of ricinoleoyl into PC as well. The inhibited interconversion of CsPDCT caused by ricinoleoyl containing DAG species is an excellent example of a potential incompatibility issue within the endogenous lipid metabolism if scientists intend to produce a seed oil rich in ricinoleic acid by gene technology. Additional measurements with increased complexity, e.g. in this case recruitment of a PDCT, not inhibited in this way, may circumvent such an incompatibility issue.

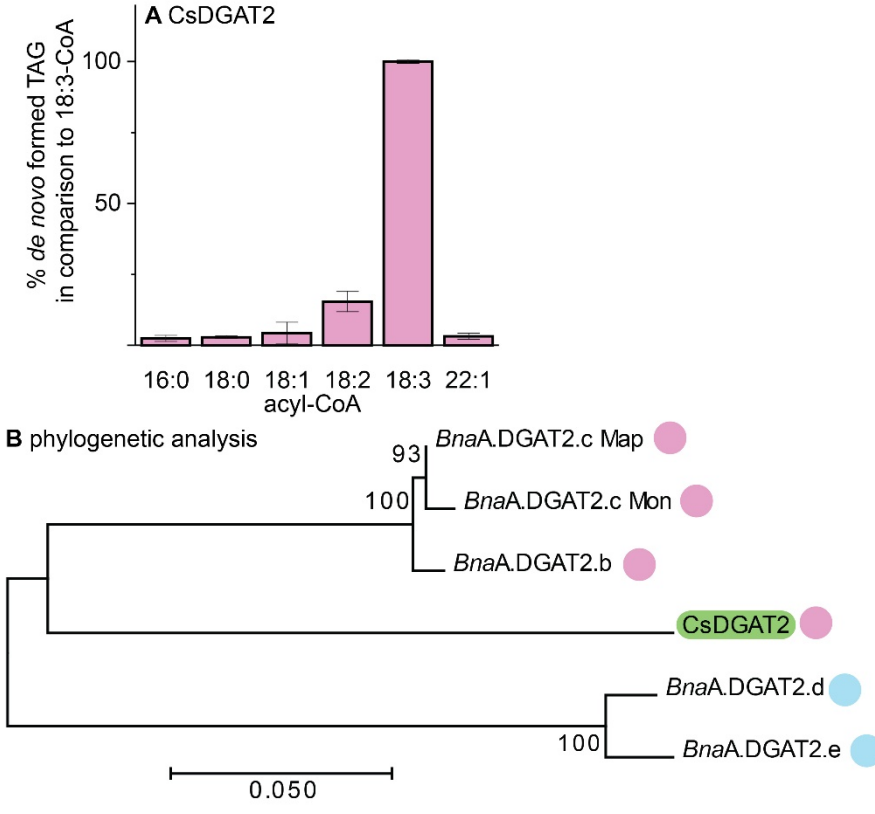

Figure 16 **A** the acyl-CoA specificity of CsDGAT2, which is similar to the acyl donor specificity of BnaA.DGAT2b (figure 11A). We assessed CsDGAT2 with di-6:0-DAG as acyl donor under close to linear conditions. Error bars denote standard deviation, n= 3. **B** phylogenetic analysis of CsDGAT2 and *B. napus* DGAT2 amino acid sequences based on the maximum likelihood method algorithm in mega X with 1000 bootstraps. A pink dot indicates a DGAT which predominantly prefers 18:3$\Delta^{9,12,15}$-CoA and a blue dot indicate an enzyme which expresses a similar preference for both 18:3$\Delta^{9,12,15}$–CoA and 22:1Δ^{13}-CoA.

5 Future perspectives and discussion

Several important questions remain unresolved despite the efforts put into this thesis, as customary. The interpretation of results from an experiment is a sometimes daunting and perplexing task for an inquisitive doctoral student, yet it is also a very inspiring and creative process. On more than one occasion, the acquired data added more questions to an ever-growing pile than it provided answers. Discussions about results and interpretation of these in the lab, with fellow researchers at other universities and scientific conferences, have always been enriching and often provided insights, previously overlooked.

We had not yet developed the methodology for assays supplemented with long-chain-DAG species during the experimental phase of **paper I**. The DGAT specificity is, therefore, merely characterised with the artificial and water-soluble acyl acceptor di-6:0-DAG. We can currently only speculate if the stark difference in acyl donor specificity will remain or not when assessed with frequently occurring natural DAG species. It would thus be beneficial to re-examine the established, starkly contrasting specificities exhibited by *B. napus* DGAT2 enzymes, with long-chain-DAG abundant in the DAG pool of developing *B. napus* seeds and the desired di-22:1Δ^{13}-DAG. These DGAT2 enzymes might not only prefer different acyl donors but may very well acylate contrasting DAG species as well, as we observed in crambe (**paper III)**.

Similarly, it would be interesting to re-evaluate the chimaeras produced during **paper II** with additional DAG-species. The acyl donor specificity is without a doubt affected by substitutions in the identified region, but what about the acyl acceptor specificity? Are the two specificities regulated by two separate regions, or by partially overlapping regions, or even within the same region? The substrate specificities, acyl acceptor and acyl donor, may also affect each other even if these reside in two separate regions as well. A stretch of amino acids

separate the putative acyl acceptor pocked motif from the predicted transmembrane helices region, and this indicates that a separate region governs the acyl acceptor specificity, see figure 13. It would also be valuable to proceed with new chimaeric enzymes to dissect the region further in the search for a more precise motif governing 22:1Δ^{13}-CoA acceptance. Examinations of additional known DGAT amino acid sequences with determined acyl donor specificities can aid in search of similar motifs promoting acylation of other acyl-CoAs as well. Such motifs may aid future *in silico* screening of candidate DGAT2 enzymes with a suitable specificity for tailormade purposes. In addition, such an identification, in turn, dependant on the size and nature of the motifs, could enable genetic engineering with, for example, CRISPR/Cas9 to change endogenous DGAT2 encoding genes altering the substrate specificity of the enzyme.

The previous biochemical characterisation of DGAT2 enzymes from several plants producing UFAs, e.g. castor bean, the tung tree and ironweed indicate that these are highly capable of incorporating their respective abundant UFA into TAG. DGAT2 enzymes are thus often mentioned as enzymes specialised in forwarding UFAs into TAG, at least in plants producing UFAs. The DGAT2 enzymes characterised in this thesis exhibit a more distinct specificity towards one or a few specific acyl-CoAs as opposed to the DGAT1 enzymes that instead accept a broader range of the assessed acyl donors at similar rates. Some of the DGAT2 enzymes investigated, prefers common PUFAs rather than abundant erucic acid, however. The extraordinary affinity towards 18:3$\Delta^{9,12,15}$-CoA coupled with the poor acceptance of 22:1Δ^{13}-CoA by the identified *T. majus* DGAT2 enzyme is therefore very interesting. What role does a DGAT2 enzyme have which strongly prefers 18:3$\Delta^{9,12,15}$ in *T. majus* which is virtually devoid of PUFAs in its seed oil?

The seed oil of *T. majus* contains the highest recorded levels of erucic acid within vascular plants, and the predominant TAG species is trierucin. A prerequisite for the synthesis of trierucin through yet known mechanisms is the acylation of di-22:1Δ^{13}-DAG with 22:1Δ^{12}-CoA. The DGAT2 enzyme of *T. majus* is entirely unable to utilise di-22:1Δ^{13}-DAG as acyl acceptor when assessed with the acyl donors included in **paper IV**. The same is true for the two investigated crambe DGAT2 in **paper III**. To our surprise, the results thus efficiently rule out DGAT2 as the enzyme catalysing the synthesis of trierucin and more or less any erucic acid transfer to TAG at all in *T. majus*.

Our data indicate that either TmPDAT, TmDGAT1, or a combination of both, are responsible for the synthesises of the abundant trierucin in *T. majus*. TmDGAT1 is as concluded here, capable of producing trierucin, albeit at reduced rates compared to the best-preferred substrates, so is the investigated crambe DGAT1 enzyme. The DGAT1 of *T. majus*, when expressed in seeds of a transgenic HEAR does increase the oil yield but not the erucic acid content and thus earlier disregarded as the producer of trierucin in *T. majus*. This may, however, be a misinterpretation as HEAR most probably already contain DGATs capable of efficiently acylating the *sn*-3 position of abundant DAG species with erucoyl-CoA. An increase of erucic acid in HEAR by expressing TmDGAT1 would have to provide at least two critical substrates, di-22:1Δ^{13}-DAG and abundant 22:1Δ^{13}-CoA. The DAG pool in common HEAR varieties are devoid of di-22:1Δ^{13}-DAG due to the inherent inability of the BnLPAT to produce di-22:1Δ^{13}-PA. Transgenic HEAR without a suitable LPAT is thus an improper system to detect the catalytic capability of producing trierucin.

The definition of UFAs is an artificial categorisation and generalisations is to be treated cautiously. UFAs are structurally diverse molecules, and their incorporation into TAG may be just as varied. DGAT2 enzymes may thus not always be the main actor in UFAs incorporation into TAG, despite the generalised assumptions based on a few observations made where DGAT2 catalyse the incorporations of UFAs into TAG.

Which mechanisms can enable such high levels of erucic acid in the seed oil of *T. majus*, as we deem the DGAT enzymes as unsuitable? TmPDAT is to the best of our knowledge still not yet characterised. Although the PC-pool in crambe, providing the acyl donor for PDAT, have deficient levels of erucic acid, it may be different in *T. majus*. How *T. majus* manage to achieve the record-high levels of erucic acid is, therefore, unfortunately, still unresolved. It is, therefore, encouraging to continue to characterise additional enzymes involved in lipid metabolism such as PDAT in order to fully understand how *T. majus* achieves this high level of erucic acid in its seed oil. Once clarified how *T. majus* is channelling erucic acid into TAG, it may be possible to implement this strategy in other oil crops such as crambe, in the pursuit of very high levels of erucic acid. The mechanisms allowing for the high levels of erucic acid in *T. majus* seed oil may, however, not be applicable in conventional oil crops. The mechanisms allowing the high levels of erucic acid might be associated with the rather low oil content in the seeds of *T. majus*, which is approximately 10 %.

The complementary specificities of the CsDGAT1 and CsDGAT2 enzymes determined in **paper V** correlates well with the seed oil composition in the transgenic lines where Cs*DGAT2* is down-regulated. The *in vitro* characterisation of these acyltransferases explain thus to a great extent the observed *in vivo* phenotypic changes in the transgenic *C. sativa*. Most of our characterisations of acyltransferases investigate one acyl donor and acyl acceptor combination at the time. The acyl-CoA- and DAG-pools in developing seeds contain a range of different species and are not homogenous as in our assays. It is yet to be determined if the acyltransferases selectivity and specificity are coherent, or whether the abundance of various substrate species affects the selectivity and specificity through competition. The characterisation of CsPDCT indicates that the presence of ricinoleoyl in DAG species affect the interconversion of other DAG species into PC. Are there similar, yet unaccounted for, effects that affect the selectivity or specificity for acyltransferases *in vivo* as well? It would, therefore, be valuable to conduct complex assays with a mixture of various DAG-species and acyl-CoA species. How to separate all the produced lipids, determine their configuration and quantify the minute quantities of them is, however a whole other story.

Living organisms are fantastic and more complex than we can comprehend. Here we investigate a small, well-defined area of interest, yet the complexity within this narrow topic is mindboggling, and there is a magnificent beauty to this complexity. Still, after 70 years of researchers' effort to understand a phenomenon, there is at least just as much waiting to be discovered.

References

Abbadi, A., Domergue, F., Bauer, J., Napier, J.A., Welti, R., Zähringer, U., Cirpus, P. & Heinz, E. (2004). Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. *The Plant Cell,* 16(10), pp. 2734-2748.

Achaya, K., Craig, B. & Youngs, C. (1964). The component fatty acids and glycerides of castor oil. *Journal of the American Oil Chemists' Society,* 41(12), pp. 783-784.

Allen, D.K., Bates, P.D. & Tjellström, H. (2015). Tracking the metabolic pulse of plant lipid production with isotopic labeling and flux analyses: Past, present and future. *Prog Lipid Res,* 58, pp. 97-120.

Andersson, M.X., Goksör, M. & Sandelius, A.S. (2007). Optical Manipulation Reveals Strong Attracting Forces at Membrane Contact Sites between Endoplasmic Reticulum and Chloroplasts. *Journal of Biological Chemistry,* 282(2), pp. 1170-1174.

Aymé, L., Arragain, S., Canonge, M., Baud, S., Touati, N., Bimai, O., Jagic, F., Louis-Mondésir, C., Briozzo, P. & Fontecave, M. (2018). Arabidopsis thaliana DGAT3 is a [2Fe-2S] protein involved in TAG biosynthesis. *Scientific reports,* 8(1), p. 17254.

Ayme, L., Baud, S., Dubreucq, B., Joffre, F. & Chardot, T. (2014). Function and Localization of the Arabidopsis thaliana Diacylglycerol Acyltransferase DGAT2 Expressed in Yeast. *Plos One,* 9(3).

Bafor, M., Jonsson, L., Stobart, A.K. & Stymne, S. (1990). Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of Cuphea lanceolata. *Biochemical Journal,* 272(1), pp. 31-38.

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. & Stymne, S. (1991). Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (Ricinus communis) endosperm. *Biochemical Journal,* 280(2), pp. 507-514.

Bates, P.D. & Browse, J. (2012). The Significance of Different Diacylgycerol Synthesis Pathways on Plant Oil Composition and Bioengineering. *Frontiers in plant science,* 3.

Bates, P.D., Durrett, T.P., Ohlrogge, J.B. & Pollard, M. (2009). Analysis of Acyl Fluxes through Multiple Pathways of Triacylglycerol Synthesis in Developing Soybean Embryos. *Plant Physiology and Biochemistry,* 150(1), pp. 55-72.

Bates, P.D., Stymne, S. & Ohlrogge, J. (2013). Biochemical pathways in seed oil synthesis. *Current opinion in plant biology,* 16(3), pp. 358-364.

Beare, J.L., Murray, T. & Campbell, J. (1957). Effects of varying proportions of dietary rapeseed oil on the rat. *Canadian Journal of Biochemistry and Physiology,* 35(12), pp. 1225-1231.

Bessoule, J.-J., Testet, E. & Cassagne, C. (1995). Synthesis of Phosphatidylcholine in the Chloroplast Envelope after Import of Lysophosphatidylcholine from Endoplasmic Reticulum Membranes. *Eur J Biochem,* 228(2), pp. 490-497.

Brough, C.L., Coventry, J.M., Christie, W.W., Kroon, J.T., Brown, A.P., Barsby, T.L. & Slabas, A.R. (1996). Towards the genetic engineering of triacylglycerols of defined fatty acid composition: major changes in erucic acid content at the sn-2 position affected by the introduction of a 1-acyl-sn-glycerol-3-phosphate acyltransferase from Limnanthes douglasii into oil seed rape. *Molecular Breeding,* 2(2), pp. 133-142.

Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I. & Browse, J. (2008). Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. *Plant Biotechnology Journal,* 6(8), pp. 819-831.
Burton, J.D., Gronwald, J.W., Somers, D.A., Gengenbach, B.G. & Wyse, D.L. (1989). Inhibition of corn acetyl-CoA carboxylase by cyclohexanedione and aryloxyphenoxypropionate herbicides. *Pesticide Biochemistry and Physiology,* 34(1), pp. 76-85.
Busson-Breysse, J., Farines, M. & Soulier, J. (1994). Jojoba wax: Its esters and some of its minor components. *Journal of the American Oil Chemists' Society,* 71(9), pp. 999-1002.
Cahoon, E.B., Cranmer, A.M., Shanklin, J. & Ohlrogge, J.B. (1994). delta 6 Hexadecenoic acid is synthesized by the activity of a soluble delta 6 palmitoyl-acyl carrier protein desaturase in Thunbergia alata endosperm. *Journal of Biological Chemistry,* 269(44), pp. 27519-27526.
Cahoon, E.B. & Kinney, A.J. (2005). The production of vegetable oils with novel properties: using genomic tools to probe and manipulate plant fatty acid metabolism. *European Journal of Lipid Science and Technology,* 107 no.5, p. 239.
Cahoon, E.B., Marillia, E.-F., Stecca, K.L., Hall, S.E., Taylor, D.C. & Kinney, A.J. (2000). Production of Fatty Acid Components of Meadowfoam Oil in Somatic Soybean Embryos. *Plant Physiol,* 124(1), pp. 243-252.
Cahoon, E.B. & Ohlrogge, J.B. (1994). Metabolic evidence for the involvement of a [delta] 4-palmitoyl-acyl carrier protein desaturase in petroselinic acid synthesis in coriander endosperm and transgenic tobacco cells. *Plant Physiol,* 104(3), pp. 827-837.
Cahoon, E.B., Ripp, K.G., Hall, S.E. & Kinney, A.J. (2001). Formation of Conjugated Δ8, Δ10-Double Bonds by Δ12-Oleic-acid Desaturase-related Enzymes BIOSYNTHETIC ORIGIN OF CALENDIC ACID. *Journal of Biological Chemistry,* 276(4), pp. 2637-2643.
Cahoon, E.B., Shanklin, J. & Ohlrogge, J.B. (1992). Expression of a coriander desaturase results in petroselinic acid production in transgenic tobacco. *Proceedings of the National Academy of Sciences,* 89(23), pp. 11184-11188.
Cao, Y.-z., Oo, K.-C. & Huang, A.H. (1990). Lysophosphatidate acyltransferase in the microsomes from maturing seeds of meadowfoam (Limnanthes alba). *Plant Physiol,* 94(3), pp. 1199-1206.
Cases, S., Smith, S.J., Zheng, Y.W., Myers, H.M., Lear, S.R., Sande, E., Novak, S., Collins, C., Welch, C.B., Lusis, A.J., Erickson, S.K. & Farese, R.V. (1998). Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proceedings of the National Academy of Sciences,* 95(22), pp. 13018-13023.
Connelly, M. & MacIntosh, S. (2018). Petition for determination of nonregulated status for DHA canola. United States Department of Agriculture: Animal and Plant Health Inspection ….
Dahlqvist, A., Ståhl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H. & Stymne, S. (2000). Phospholipid: diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proceedings of the National Academy of Sciences,* 97(12), pp. 6487-6492.
Dewey, R.E., Wilson, R.F., Novitzky, W.P. & Goode, J.H. (1994). The AAPT1 gene of soybean complements a cholinephosphotransferase-deficient mutant of yeast. *The Plant Cell,* 6(10), pp. 1495-1507.
Diederichsen, A. & Hammer, K. (2003). The infraspecific taxa of coriander (Coriandrum sativum L.). *Genetic Resources Crop Evolution,* 50(1), pp. 33-63.
Dorne, A.J., Joyard, J. & Douce, R. (1990). Do thylakoids really contain phosphatidylcholine? *Proceedings of the National Academy of Sciences,* 87(1), pp. 71-74.
Downey, R.K. & Harvey, B.L. (1963). Methods of breeding for oil quality in rape. *Canadian Journal of Plant Science,* 43(3), pp. 271-275.
Dubois, V., Breton, S., Linder, M., Fanni, J. & Parmentier, M. (2007). Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. *European Journal of Lipid Science and Technology,* 109(7), pp. 710-732.
Dyer, J.M., Chapital, D.C., Kuan, J.-C.W., Mullen, R.T., Turner, C., McKeon, T.A. & Pepperman, A.B. (2002). Molecular analysis of a bifunctional fatty acid conjugase/desaturase from tung. Implications for the evolution of plant fatty acid diversity. *Plant Physiol,* 130(4), pp. 2027-2038.
Eastmond, P.J., Quettier, A.-L., Kroon, J.T.M., Craddock, C., Adams, N. & Slabas, A.R. (2010). PHOSPHATIDIC ACID PHOSPHOHYDROLASE1 and 2 Regulate Phospholipid Synthesis at the Endoplasmic Reticulum in Arabidopsis. *The Plant Cell,* 22(8), pp. 2796-2811.

FitzJohn, R.G., Armstrong, T.T., Newstrom-Lloyd, L.E., Wilton, A.D. & Cochrane, M. (2007). Hybridisation within Brassica and allied genera: evaluation of potential for transgene escape. *Euphytica,* 158(1-2), pp. 209-230.
Fourmann, M., Barret, P., Renard, M., Pelletier, G., Delourme, R. & Brunel, D. (1998). The two genes homologous to Arabidopsis FAE1 co-segregate with the two loci governing erucic acid content in Brassica napus. *Theoretical and Applied Genetics,* 96(6-7), pp. 852-858.
Furmanek, T., Demski, K., Banas, W., Haslam, R., Napier, J., Stymne, S. & Banas, A. (2014). The Utilization of the Acyl-CoA and the Involvement PDAT and DGAT in the Biosynthesis of Erucic Acid-Rich Triacylglycerols in Crambe Seed Oil. *Lipids,* 49(4), pp. 327-333.
Gentry, H.S. (1958). The natural history of jojoba (Simmondsia chinensis) and its cultural aspects. *Economic botany,* 12(3), pp. 261-295.
Gląb, B., Beganovic, M., Anaokar, S., Hao, M.-S., Rasmusson, A., Patton-Vogt, J., Banaś, A., Stymne, S. & Lager, I. (2016). Cloning of glycerophosphocholine acyltransferase (GPCAT) from fungi and plants; a novel enzyme in phosphatidylcholine synthesis. *Journal of Biological Chemistry,* 291, no. 48, pp. 25066-25076.
Griffiths, G. & Harwood, J.L. (1991). The regulation of triacylglycerol biosynthesis in cocoa (Theobroma cacao) L. *Planta,* 184(2), pp. 279-284.
Griffiths, G., Stymne, S. & Stobart, A.K. (1988). Phosphatidylcholine and its relationship to triacylglycerol biosynthesis in oil-tissues*,* 27(7), pp. 2089-2093.
Grimberg, Å., Carlsson, A.S., Marttila, S., Bhalerao, R. & Hofvander, P. (2015). Transcriptional transitions in Nicotiana benthamiana leaves upon induction of oil synthesis by WRINKLED1 homologs from diverse species and tissues. *BMC Plant Biology,* 15(1).
Guan, R., Lager, I., Li, X.Y., Stymne, S. & Zhu, L.H. (2014). Bottlenecks in erucic acid accumulation in genetically engineered ultrahigh erucic acid Crambe abyssinica. *Plant Biotechnology Journal,* 12(2), pp. 193-203.
Guerra, D.J. & Ohlrogge, J.B. (1986). Partial purification and characterization of two forms of malonyl-coenzyme A:Acyl carrier protein transacylase from soybean leaf tissue. *Arch Biochem Biophys,* 246(1), pp. 274-285.
Halleux, H., Lassaux, S., Renzoni, R. & Germain, A. (2008). Comparative life cycle assessment of two biofuels ethanol from sugar beet and rapeseed methyl ester. *The International Journal of Life Cycle Assessment,* 13(3), pp. 184-190.
Han, J., Lühs, W., Sonntag, K., Zähringer, U., Borchardt, D.S., Wolter, F.P., Heinz, E. & Frentzen, M. (2001). Functional characterization of β-ketoacyl-CoA synthase genes from Brassica napus L. *Plant Mol Biol,* 46(2), pp. 229-239.
Harris, W.S. (2004). Fish oil supplementation: evidence for health benefits. *Cleveland Clinic journal of medicine,* 71(3), pp. 208-221.
Harvey, B. & Downey, R. (1964). The inheritance of erucic acid content in rapeseed (Brassica napus). *Canadian Journal of Plant Science,* 44(1), pp. 104-111.
Harwood, J.L. (1988). Fatty acid metabolism. *Annual Review of Plant Physiology,* 39(1), pp. 101-138.
Harwood, J.L. (1996). Recent advances in the biosynthesis of plant fatty acids. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism,* 1301(1-2), pp. 7-56.
Heilmann, I., Mekhedov, S., King, B. & Shanklin, J. (2004). Identification of the Arabidopsis palmitoyl-monogalactosyldiacylglycerol Δ7-desaturase gene FAD5, and effects of plastidial retargeting of Arabidopsis desaturases on the fad5 mutant phenotype. *Plant Physiol,* 136(4), pp. 4237-4245.
Heinz, E. & Roughan, P.G. (1983). Similarities and differences in lipid metabolism of chloroplasts isolated from 18: 3 and 16: 3 plants. *Plant Physiol,* 72(2), pp. 273-279.
Hopkins, C., Chisholm, M.J. & Prince, L. (1966). Fatty acids ofLindera umbellata and other Lauraceae seed oils. *Lipids,* 1(2), pp. 118-122.
Huang, W., Jia, J., Edwards, P., Dehesh, K., Schneider, G. & Lindqvist, Y. (1998). Crystal structure of β-ketoacyl-acyl carrier protein synthase II from E. coli reveals the molecular architecture of condensing enzymes. *The EMBO journal,* 17(5), pp. 1183-1191.
Iorizzo, M., Senalik, D.A., Ellison, S.L., Grzebelus, D., Cavagnaro, P.F., Allender, C., Brunet, J., Spooner, D.M., Van Deynze, A. & Simon, P.W. (2013). Genetic structure and domestication of carrot (Daucus carota subsp. sativus) (Apiaceae). *American Journal of Botany,* 100(5), pp. 930-938.
Jako, C., Kumar, A., Wei, Y., Zou, J., Barton, D.L., Giblin, E.M., Covello, P.S. & Taylor, D.C. (2001). Seed-specific over-expression of an Arabidopsis cDNA encoding a diacylglycerol

acyltransferase enhances seed oil content and seed weight. *Plant Physiol,* 126(2), pp. 861-874.
Jiang, C., Kim, S.Y. & Suh, D.-Y. (2008). Divergent evolution of the thiolase superfamily and chalcone synthase family. *Molecular phylogenetics and evolution,* 49(3), pp. 691-701.
Jones, A. (1995). Palmitoyl-Acyl Carrier Protein (ACP) Thioesterase and the Evolutionary Origin of Plant Acyl-ACP Thioesterases. *The Plant Cell,* 7(3), pp. 359-371.
Kannangara, C.G. & Stumpf, P.K. (1972). Fat metabolism in higher plants. *Arch Biochem Biophys,* 152(1), pp. 83-91.
Karki, N., Johnson, B.S. & Bates, P.D. (2019). Metabolically distinct pools of phosphatidylcholine are involved in trafficking of fatty acids out of and into the chloroplast for membrane production. *The Plant Cell,* 31(11), pp. 2768-2788.
Katavic, V., Reed, D.W., Taylor, D.C., Giblin, E.M., Barton, D.L., Zou, J., MacKenzie, S.L., Covello, P.S. & Kunst, L. (1995). Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in Arabidopsis thaliana affecting diacylglycerol acyltransferase activity. *Plant Physiol,* 108(1), pp. 399-409.
Kjellberg, J.M., Trimborn, M., Andersson, M. & Sandelius, A.S. (2000). Acyl-CoA dependent acylation of phospholipids in the chloroplast envelope. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1485(2-3), pp. 100-110.
Kleiman, R. & Spencer, G.F. (1982). Search for new industrial oils: XVI. Umbelliflorae—seed oils rich in petroselinic acid. *Journal of the American Oil Chemists' Society,* 59(1), pp. 29-38.
Knutzon, D.S., Lardizabal, K.D., Nelsen, J.S., Bleibaum, J.L., Davies, H.M. & Metz, J. (1995). Cloning of a coconut endosperm cDNA encoding a 1-acyl-sn-glycerol-3-phosphate acyltransferase that accepts medium-chain-length substrates. *Plant Physiology and Biochemistry,* 109(3), pp. 999-1006.
Kobayashi, M., Msangi, S., Batka, M., Vannuccini, S., Dey, M.M. & Anderson, J.L. (2015). Fish to 2030: the role and opportunity for aquaculture. *Aquaculture economics & management,* 19(3), pp. 282-300.
Konishi, T., Shinohara, K., Yamada, K. & Sasaki, Y. (1996). Acetyl-CoA Carboxylase in Higher Plants: Most Plants Other Than Gramineae Have Both the Prokaryotic and the Eukaryotic Forms of This Enzyme. *Plant and Cell Physiology,* 37(2), pp. 117-122.
Lager, I., Yilmaz, J.L., Zhou, X.-R., Jasieniecka, K., Kazachkov, M., Wang, P., Zou, J., Weselake, R., Smith, M.A. & Bayon, S. (2013). Plant acyl-CoA: lysophosphatidylcholine acyltransferases (LPCATs) have different specificities in their forward and reverse reactions. *Journal of Biological Chemistry,* 288(52), pp. 36902-36914.
Lardizabal, K.D., Mai, J.T., Wagner, N.W., Wyrick, A., Voelker, T. & Hawkins, D.J. (2001). DGAT2 is a new diacylglycerol acyltransferase gene family Purification, cloning, and expression in insect cells of two polypeptides from Mortierella ramanniana with diacylglycerol acyltransferase activity. *Journal of Biological Chemistry,* 276(42), pp. 38862-38869.
Lassner, M.W., Levering, C.K., Davies, H.M. & Knutzon, D.S. (1995). Lysophosphatidic acid acyltransferase from meadowfoam mediates insertion of erucic acid at the sn-2 position of triacylglycerol in transgenic rapeseed oil. *Plant Physiol,* 109(4), pp. 1389-1394.
Lazzeri, L., De Mattei, F., Bucelli, F. & Palmieri, S. (1997). Crambe oil-a potential new hydraulic oil and quenchant. *Industrial Lubrication and Tribology,* 49(2), pp. 71-77.
Lee, M., Lenman, M., Banaś, A., Bafor, M., Singh, S., Schweizer, M., Nilsson, R., Liljenberg, C., Dahlqvist, A. & Gummeson, P.-O. (1998). Identification of non-heme diiron proteins that catalyze triple bond and epoxy group formation. *Science,* 280(5365), pp. 915-918.
Leonard, J.M., Knapp, S.J. & Slabaugh, M.B. (1998). A Cupheabeta-ketoacyl-ACP synthase shifts the synthesis of fatty acids towards shorter chains in Arabidopsis seeds expressing Cuphea FatB thioesterases. *The Plant Journal,* 13(5), pp. 621-628.
Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., Arondel, V., Bates, P.D., Baud, S., Bird, D., Debono, A., Durrett, T.P., Franke, R.B., Graham, I.A., Katayama, K., Kelly, A.A., Larson, T., Markham, J.E., Miquel, M., Molina, I., Nishida, I., Rowland, O., Samuels, L., Schmid, K.M., Wada, H., Welti, R., Xu, C., Zallot, R. & Ohlrogge, J. (2010). Acyl-Lipid Metabolism. *The Arabidopsis Book,* 8, p. e0133.
Li, N., Gügel, I.L., Giavalisco, P., Zeisler, V., Schreiber, L., Soll, J. & Philippar, K. (2015). FAX1, a Novel Membrane Protein Mediating Plastid Fatty Acid Export. *PLOS Biology,* 13(2), p. e1002053.
Li, R., Yu, K., Hatanaka, T. & Hildebrand, D.F. (2010a). Vernonia DGATs increase accumulation of epoxy fatty acids in oil. *Plant Biotechnology Journal,* 8(2), pp. 184-195.

Li, R., Yu, K. & Hildebrand, D.F. (2010b). DGAT1, DGAT2 and PDAT Expression in Seeds and Other Tissues of Epoxy and Hydroxy Fatty Acid Accumulating Plants. *Lipids,* 45(2), pp. 145-157.
Li, X., Teitgen, A.M., Shirani, A., Ling, J., Busta, L., Cahoon, R.E., Zhang, W., Li, Z., Chapman, K.D. & Berman, D. (2018). Discontinuous fatty acid elongation yields hydroxylated seed oil with improved function. *Nature plants,* 4(9), pp. 711-720.
Li, X., van Loo, E.N., Gruber, J., Fan, J., Guan, R., Frentzen, M., Stymne, S. & Zhu, L.H. (2012). Development of ultra-high erucic acid oil in the industrial oil crop Crambe abyssinica. *Plant Biotechnology Journal,* 10(7), pp. 862-870.
Liu, Q., Siloto, R.M., Snyder, C.L. & Weselake, R.J. (2011). Functional and topological analysis of yeast acyl-CoA: diacylglycerol acyltransferase 2, an endoplasmic reticulum enzyme essential for triacylglycerol biosynthesis. *Journal of Biological Chemistry,* 286(15), pp. 13115-13126.
Lu, C., Xin, Z., Ren, Z., Miquel, M. & Browse, J. (2009). An enzyme regulating triacylglycerol composition is encoded by the ROD1 gene of Arabidopsis. *Proceedings of the National Academy of Sciences,* 106(44), pp. 18837-18842.
Lu, S., Wang, J., Chitsaz, F., Derbyshire, M.K., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Marchler, G.H. & Song, J.S. (2020). CDD/SPARCLE: the conserved domain database in 2020. *Nucleic Acids Res,* 48(D1), pp. D265-D268.
Löhden, I. & Frentzen, M. (1992). Triacylglycerol biosynthesis in developing seeds of Tropaeolum majus L. and Limnanthes douglasii R. Br. *Planta,* 188(2), pp. 215-224.
Malcicka, M., Visser, B. & Ellers, J. (2018). An evolutionary perspective on linoleic acid synthesis in animals. *Evolutionary biology,* 45(1), pp. 15-26.
Marmon, S., Sturtevant, D., Herrfurth, C., Chapman, K., Stymne, S. & Feussner, I. (2017). Two acyltransferases contribute differently to linolenic acid levels in seed oil. *Plant Physiol,* 173(4), pp. 2081-2095.
Matthaus, B., Vosmann, K., Pham, L.Q. & Aitzetmüller, K. (2003). FA and tocopherol composition of Vietnamese oilseeds. *Journal of the American Oil Chemists' Society,* 80(10), pp. 1013-1020.
McKeon, T.A. & Stumpf, P. (1982). Purification and characterization of the stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower. *Journal of Biological Chemistry,* 257(20), pp. 12141-12147.
McVetty, P.B., Mietkiewska, E., Omonov, T., Curtis, J., Taylor, D.C. & Weselake, R.J. (2016). Brassica spp. oils. In: *Industrial Oil Crops* Elsevier, pp. 113-156.
McVetty, P.B. & Scarth, R. (2002). Breeding for improved oil quality in Brassica oilseed species. *Journal of crop production,* 5(1-2), pp. 345-369.
Metz, J.G., Kuner, J., Rosenzweig, B., Lippmeier, J.C., Roessler, P. & Zirkle, R. (2009). Biochemical characterization of polyunsaturated fatty acid synthesis in Schizochytrium: release of the products as free fatty acids. *Plant Physiology and Biochemistry,* 47(6), pp. 472-478.
Mietkiewska, E., Brost, J.M., Giblin, E.M., Barton, D.L. & Taylor, D.C. (2007). Cloning and functional characterization of the fatty acid elongase 1 (FAE1) gene from high erucic Crambe abyssinica cv. Prophet. *Plant Biotechnology Journal,* 5(5), pp. 636-645.
Mietkiewska, E., Giblin, E.M., Wang, S., Barton, D.L., Dirpaul, J., Brost, J.M., Katavic, V. & Taylor, D.C. (2004). Seed-specific heterologous expression of a nasturtium FAE gene in Arabidopsis results in a dramatic increase in the proportion of erucic acid. *Plant Physiol,* 136(1), pp. 2665-2675.
Millar, A.A. & Kunst, L. (1997). Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *The Plant Journal,* 12(1), pp. 121-131.
Millar, A.A., Smith, M.A. & Kunst, L. (2000). All fatty acids are not equal: discrimination in plant membrane lipids. *Trends in plant science,* 5(3), pp. 95-101.
Mongrand, S., Bessoule, J.-J., Cabantous, F. & Cassagne, C.J.P. (1998). The C16: 3\C18: 3 fatty acid balance in photosynthetic tissues from 468 plant species, 49(4), pp. 1049-1064.
Moreau, R.A., Pollard, M.R. & Stumpf, P.K. (1981). Properties of a Δ5-fatty acyl-CoA desaturase in the cotyledons of developing Limnanthes alba. *Arch Biochem Biophys,* 209(2), pp. 376-384.
Moreau, R.A. & Stumpf, P.K. (1981). Recent Studies of the Enzymic Synthesis of Ricinoleic Acid by Developing Castor Beans. *Plant Physiol,* 67(4), pp. 672-676.
Murphy, D.J. (1992). Identification and characterisation of genes and enzymes for the genetic engineering of oilseed crops for production of oils for the oleochemical industry: a review. *Industrial CropsProducts,* 1(2-4), pp. 251-259.
Napier, J.A., Olsen, R.E. & Tocher, D.R. (2019). Update on GM canola crops as novel sources of omega-3 fish oils. *Plant Biotechnology Journal,* 17(4), p. 703.

Napier, J.A., Usher, S., Haslam, R.P., Ruiz-Lopez, N. & Sayanova, O. (2015). Transgenic plants as a sustainable, terrestrial source of fish oils. *European Journal of Lipid Science and Technology,* 117(9), pp. 1317-1324.
Nath, U.K., Wilmer, J.A., Wallington, E.J., Becker, H.C. & Möllers, C. (2009). Increasing erucic acid content through combination of endogenous low polyunsaturated fatty acids alleles with Ld-LPAAT+ Bn-fae1 transgenes in rapeseed (Brassica napus L.). *Theoretical and Applied Genetics,* 118(4), pp. 765-773.
Neff, W.E. & El-Agaimy, M. (1996). Effect of Linoleic Acid Position in Triacylglycerols on their Oxidative Stability. *Lebensmittel-Wissenschaft und Technologie,* 29(8), pp. 772-775.
Ogunniyi, D. (2006). Castor oil: A vital industrial raw material. *Bioresource Technology,* 97(9), pp. 1086-1091.
Ohlrogge, J. & Browse, J. (1995). Lipid biosynthesis. *The Plant Cell,* 7(7), pp. 957-970.
Ohlrogge, J., Thrower, N., Mhaske, V., Stymne, S., Baxter, M., Yang, W., Liu, J., Shaw, K., Shorrosh, B. & Zhang, M. (2018). Plant FA db: a resource for exploring hundreds of plant fatty acid structures synthesized by thousands of plants and their phylogenetic relationships. *The Plant Journal,* 96(6), pp. 1299-1308.
Ohlrogge, J.B., Kuhn, D.N. & Stumpf, P.K. (1979). Subcellular localization of acyl carrier protein in leaf protoplasts of Spinacia oleracea. *Proceedings of the National Academy of Sciences,* 76(3), pp. 1194-1198.
Ortiz, R., Geleta, M., Gustafsson, C., Lager, I., Hofvander, P., Löfstedt, C., Cahoon, E.B., Minina, E., Bozhkov, P. & Stymne, S. (2020). Oil crops for the future. *Current opinion in plant biology*.
Petrie, J.R., Shrestha, P., Belide, S., Kennedy, Y., Lester, G., Liu, Q., Divi, U.K., Mulder, R.J., Mansour, M.P. & Nichols, P.D. (2014). Metabolic engineering Camelina sativa with fish oil-like levels of DHA. *Plos One,* 9(1).
Petrie, J.R. & Singh, S.P. (2011). Expanding the docosahexaenoic acid food web for sustainable production: engineering lower plant pathways into higher plants. *AoB Plants,* 2011.
Piazza, G.J. & Foglia, T.A. (2001). Rapeseed oil for oleochemical usage. *European Journal of Lipid Science and Technology,* 103(7), pp. 450-454.
Qi, B., Fraser, T., Mugford, S., Dobson, G., Sayanova, O., Butler, J., Napier, J.A., Stobart, A.K. & Lazarus, C.M. (2004). Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. *Nature biotechnology,* 22(6), pp. 739-745.
Roughan, G. & Slack, R. (1984). Glycerolipid synthesis in leaves. *Trends in Biochemical Sciences,* 9(9), pp. 383-386.
Routaboul, J.-M., Benning, C., Bechtold, N., Caboche, M. & Lepiniec, L. (1999). The TAG1 locus of Arabidopsis encodes for a diacylglycerol acyltransferase. *Plant Physiology and Biochemistry,* 37(11), pp. 831-840.
Ruiz-Lopez, N., Haslam, R.P., Napier, J.A. & Sayanova, O. (2014). Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. *The Plant Journal,* 77(2), pp. 198-208.
Saha, S., Enugutti, B., Rajakumari, S. & Rajasekharan, R. (2006). Cytosolic triacylglycerol biosynthetic pathway in oilseeds. Molecular cloning and expression of peanut cytosolic diacylglycerol acyltransferase. *Plant Physiol,* 141(4), pp. 1533-1543.
Sandager, L., Gustavsson, M.H., Stahl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H. & Stymne, S. (2002). Storage lipid synthesis is non-essential in yeast. *Journal of Biological Chemistry,* 277(8), pp. 6478-6482.
Sayanova, O., Haslam, R., Venegas Caleron, M. & Napier, J.A. (2007). Cloning and Characterization of Unusual Fatty Acid Desaturases from Anemone leveillei: Identification of an Acyl-Coenzyme A C20 Δ5-Desaturase Responsible for the Synthesis of Sciadonic Acid. *Plant Physiol,* 144(1), pp. 455-467.
Scarth, R. & Tang, J. (2006). Modification of Brassica oil using conventional and transgenic approaches. *Crop science,* 46(3), pp. 1225-1236.
Schnurr, J.A. (2002). Fatty Acid Export from the Chloroplast. Molecular Characterization of a Major Plastidial Acyl-Coenzyme A Synthetase from Arabidopsis. *Plant Physiology and Biochemistry,* 129(4), pp. 1700-1709.
Shanklin, J. & Cahoon, E.B. (1998). Desaturation and related modifications of fatty acids. *Annual Review of Plant Biology,* 49(1), pp. 611-641.
Shi, J., Lang, C., Wang, F., Wu, X., Liu, R., Zheng, T., Zhang, D., Chen, J. & Wu, G. (2017). Depressed expression of FAE1 and FAD2 genes modifies fatty acid profiles and storage compounds accumulation in Brassica napus seeds. *Plant Science,* 263, pp. 177-182.

Shirani, A., Joy, T., Lager, I., Yilmaz, J.L., Wang, H.-L., Jeppson, S., Cahoon, E.B., Chapman, K., Stymne, S. & Berman, D. (2020). Lubrication characteristics of wax esters from oils produced by a genetically-enhanced oilseed crop. *Tribology International,* 146, p. 106234.
Shockey, J.M., Gidda, S.K., Chapital, D.C., Kuan, J.-C., Dhanoa, P.K., Bland, J.M., Rothstein, S.J., Mullen, R.T. & Dyer, J.M. (2006). Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. *The Plant Cell,* 18(9), pp. 2294-2313.
Slabas, A.R. & Fawcett, T. (1992). The biochemistry and molecular biology of plant lipid biosynthesis. *Plant Mol Biol,* 19(1), pp. 169-191.
Slack, C.R., Roughan, P.G. & Balasingham, N. (1978). Labelling of glycerolipids in the cotyledons of developing oilseeds by [1-14C] acetate and [2-3H] glycerol. *Biochemical Journal,* 170(2), pp. 421-433.
Slack, C.R., Roughan, P.G., Browse, J.A. & Gardiner, S.E. (1985). Some properties of cholinephosphotransferase from developing safflower cotyledons. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 833(3), pp. 438-448.
Smith, M.A., Dauk, M., Ramadan, H., Yang, H., Seamons, L.E., Haslam, R.P., Beaudoin, F., Ramirez-Erosa, I. & Forseille, L. (2013). Involvement of Arabidopsis ACYL-COENZYME A DESATURASE-LIKE2 (At2g31360) in the Biosynthesis of the Very-Long-Chain Monounsaturated Fatty Acid Components of Membrane Lipids. *Plant Physiol,* 161(1), pp. 81-96.
Snell, P., Grimberg, Å., Carlsson, A.S. & Hofvander, P. (2019). WRINKLED1 Is Subject to Evolutionary Conserved Negative Autoregulation. *Frontiers in plant science,* 10.
Sottosanto, J., Andre, C., Arias, D., Bhatti, M., Breazeale, S., Fu, H. & Klucinec, A. (2018). *Petition for the determination of nonregulatory status for EPA+ DHA canola event LBFLFK*.
Stone, S.J., Levin, M.C. & Farese, R.V. (2006). Membrane topology and identification of key functional amino acid residues of murine acyl-CoA: diacylglycerol acyltransferase-2. *Journal of Biological Chemistry,* 281(52), pp. 40273-40282.
Sturtevant, D., Lee, Y.-J. & Chapman, K.D. (2016). Matrix assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) for direct visualization of plant metabolites in situ. *Current Opinion in Biotechnology,* 37, pp. 53-60.
Taylor, D.C., Francis, T., Lozinsky, S., Hoffman, T., Giblin, M. & Marillia, E.-F. (2010). Cloning and characterization of a constitutive lysophosphatidic acid acyltransferase 2 (LPAT2) gene from Tropaeolum majus L. *The Open Plant Science Journal,* 4(1).
Taylor, D.C., MacKenzie, S.L., McCurdy, A.R., McVetty, P.B., Giblin, E.M., Pass, E.W., Stone, S.J., Scarth, R., Rimmer, S.R. & Pickard, M.D. (1994). Stereospecific analyses of seed triacylglycerols from high-erucic acid brassicaceae: Detection of erucic acid at thesn-2 position inBrassica oleracea L. Genotypes. *Journal of the American Oil Chemists' Society,* 71(2), pp. 163-167.
Thompson, A. & Kleiman, R. (1988). Effect of seed maturity on seed oil, fatty acid and crude protein content of eight Cuphea species. *Journal of the American Oil Chemists' Society,* 65(1), pp. 139-146.
Tjellström, H., Yang, Z., Allen, D.K. & Ohlrogge, J.B. (2012). Rapid kinetic labeling of Arabidopsis cell suspension cultures: implications for models of lipid export from plastids. *Plant Physiology and Biochemistry,* 158(2), pp. 601-611.
Tocher, D.R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture,* 449, pp. 94-107.
Troncoso-Ponce, M.A., Nikovics, K., Marchive, C., Lepiniec, L. & Baud, S. (2016). New insights on the organization and regulation of the fatty acid biosynthetic network in the model higher plant Arabidopsis thaliana. *Biochimie,* 120, pp. 3-8.
Turchetto-Zolet, A.C., Maraschin, F.S., de Morais, G.L., Cagliari, A., Andrade, C.M., Margis-Pinheiro, M. & Margis, R. (2011). Evolutionary view of acyl-CoA diacylglycerol acyltransferase (DGAT), a key enzyme in neutral lipid biosynthesis. *BMC evolutionary biology,* 11(1), p. 263.
Turnbull, A.P., Rafferty, J.B., Sedelnikova, S.E., Slabas, A.R., Schierer, T.P., Kroon, J.T., Simon, J.W., Fawcett, T., Nishida, I. & Murata, N. (2001). Analysis of the structure, substrate specificity, and mechanism of squash glycerol-3-phosphate (1)-acyltransferase. *Structure,* 9(5), pp. 347-353.
Usher, S., Han, L., Haslam, R.P., Michaelson, L.V., Sturtevant, D., Aziz, M., Chapman, K.D., Sayanova, O. & Napier, J.A. (2017). Tailoring seed oil composition in the real world:

optimising omega-3 long chain polyunsaturated fatty acid accumulation in transgenic Camelina sativa. *Scientific reports,* 7(1), pp. 1-12.
Walsh, T.A., Bevan, S.A., Gachotte, D.J., Larsen, C.M., Moskal, W.A., Merlo, P.O., Sidorenko, L.V., Hampton, R.E., Stoltz, V. & Pareddy, D. (2016). Canola engineered with a microalgal polyketide synthase-like system produces oil enriched in docosahexaenoic acid. *Nature biotechnology,* 34(8), p. 881.
Van De Loo, F.J., Broun, P., Turner, S. & Somerville, C. (1995). An oleate 12-hydroxylase from Ricinus communis L. is a fatty acyl desaturase homolog. *Proceedings of the National Academy of Sciences,* 92(15), pp. 6743-6747.
Warner, D.J. & Lewis, K.A. (2019). Evaluation of the risks of contaminating low erucic acid rapeseed with high erucic rapeseed and identification of mitigation strategies. *Agriculture,* 9(9), p. 190.
Weier, D., Hanke, C., Eickelkamp, A., Lühs, W., Dettendorfer, J., Schaffert, E., Möllers, C., Friedt, W., Wolter, F.P. & Frentzen, M. (1997). Trierucoylglycerol biosynthesis in transgenic plants of rapeseed (Brassica napus L.). *Lipid/Fett,* 99(5), pp. 160-165.
Weiss, S.B. & Kennedy, E.P. (1956). The enzymatic synthesis of triglycerides. *Journal of the American Chemical Society,* 78(14), pp. 3550-3550.
Weiss, S.B., Kennedy, E.P. & Kiyasu, J.Y.J.J.o.B.C. (1960). The enzymatic synthesis of triglycerides*,* 235(1), pp. 40-44.
Voelker, T. & Kinney, A.J. (2001). V ARIATIONS IN THE B IOSYNTHESIS OF S EED -S TORAGE L IPIDS*,* 52(1), pp. 335-361.
Voelker, T.A., Hayes, T.R., Cranmer, A.M., Turner, J.C. & Davies, H.M. (1996). Genetic engineering of a quantitative trait: metabolic and genetic parameters influencing the accumulation of laurate in rapeseed. *The Plant Journal,* 9(2), pp. 229-241.
von Wettstein-Knowles, P. (1982). Elongase and epicuticular wax biosynthesis. *Physiol Veg,* 20, pp. 797-809.
Woodfield, H.K., Sturtevant, D., Borisjuk, L., Munz, E., Guschina, I.A., Chapman, K. & Harwood, J.L. (2017). Spatial and temporal mapping of key lipid species in Brassica napus seeds. *J Plant physiology,* 173(4), pp. 1998-2009.
Xu, J., Francis, T., Mietkiewska, E., Giblin, E.M., Barton, D.L., Zhang, Y., Zhang, M. & Taylor, D.C. (2008). Cloning and characterization of an acyl-CoA-dependent diacylglycerol acyltransferase 1 (DGAT1) gene from Tropaeolum majus, and a study of the functional motifs of the DGAT protein using site-directed mutagenesis to modify enzyme activity and oil content. *Plant Biotechnology Journal,* 6(8), pp. 799-818.
Ye, Y., Cochrane, K., Bianchi, G., Willmann, R., Majkowski, J., Tandstad, M. & Carocci, F. (2013). Rebuilding global fisheries: the World Summit Goal, costs and benefits. *Fish and Fisheries,* 14(2), pp. 174-185.
Yen, C.-L.E., Stone, S.J., Koliwad, S., Harris, C. & Farese, R.V. (2008). Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J Lipid Res,* 49(11), pp. 2283-2301.
Zhang, M., Fan, J., Taylor, D.C. & Ohlrogge, J.B. (2009). DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. *The Plant Cell,* 21(12), pp. 3885-3901.
Zhao, L., Katavic, V., Li, F., Haughn, G.W. & Kunst, L. (2010). Insertional mutant analysis reveals that long-chain acyl-CoA synthetase 1 (LACS1), but not LACS8, functionally overlaps with LACS9 in Arabidopsis seed oil biosynthesis. *The Plant Journal,* 64(6), pp. 1048-1058.
Zheng, Z. (2003). Arabidopsis AtGPAT1, a Member of the Membrane-Bound Glycerol-3-Phosphate Acyltransferase Gene Family, Is Essential for Tapetum Differentiation and Male Fertility. *THE PLANT CELL ONLINE,* 15(8), pp. 1872-1887.
Zhou, X.-R., Shrestha, P., Yin, F., Petrie, J.R. & Singh, S.P. (2013). AtDGAT2 is a functional acyl-CoA: diacylglycerol acyltransferase and displays different acyl-CoA substrate preferences than AtDGAT1. *Febs Letters,* 587(15), pp. 2371-2376.

Popular science summary

Plants are incredible organisms that can produce seed oils suitable for human consumption. Seed oils can also replace fossil oil-based products as an environmentally friendly alternative. Plant breeders and researchers continually improve not only the oil yield but also the oil quality. Industrial applications demand particular oil qualities and the more suitable oil a plant can produce, the more competitive the environmentally friendly alternative becomes. Plants produce oils with small building blocks, and these are later modified and put together to oil. Specific proteins or enzymes are responsible for the production of the seed oil. These enzymes often prefer specific building blocks, and there are many of these in a plant. Enzymes preferences for building blocks is called substrate specificity. Each plant has its variants of enzymes, and these enzymes often have slightly different specificities. To improve or change the oil quality in plants, one has to know how the oil-producing enzymes will react in order to be successful. We have therefore investigated the preference of several enzymes specifically for erucic acid, a constituent in some seed oils, used in many industrial applications. During our investigations, we have understood the importance of enzymes specificities and that it due to these may be challenging to enhance the content of erucic acid in plant oils. DGAT2 is an enzyme that is important in the final production of oil. Enzymes are large molecules made by subunits called amino acids connected in a long chain. These amino acids determine the behaviour of enzymes, and thus, their substrate specificity. Through changing some of the amino acids in DGAT2, we have managed to change enzymes that dislike erucic acid into enzymes that readily accept erucic acid. The detailed studies of enzymes involved in the production of oil will aid the pursuit of better-adapted oils.

Populärvetenskaplig sammanfattning

Växter är otroliga organismer och kan producera fröoljor som är lämpliga för konsumtion. Fröoljor kan också ersätta fossila oljebaserade produkter som ett miljövänligt alternativ. Växtförädlare och forskare förbättrar kontinuerligt inte bara oljeavkastningen utan även oljekvaliteten. Industriella tillämpningar kräver särskilda oljekvaliteter och ju mer lämplig olja en växt kan producera, desto mer konkurrenskraftigt blir det miljövänligare alternativet. Växter producerar oljor med hjälp av små byggstenar, dessa modifieras senare och sätts ihop till olja. Särskilda proteiner eller enzymer ansvarar för produktionen av fröoljan. Dessa enzymer föredrar ofta specifika byggstenar, och det finns många av dessa i en växt. Enzymers preferenser för de olika byggstenarna kallas substratspecificitet. Varje växt har sina varianter av enzymer, och dessa enzymer har ofta något olika specificiteter. För att förbättra eller ändra oljekvaliteten i växter måste man därför veta hur de oljeproducerande enzymerna kommer att reagera för att lyckas. Vi har därför undersökt preferensen för flera enzymer specifikt för erukasyra, en beståndsdel i vissa fröoljor, som används i många industriella tillämpningar. Under våra undersökningar har vi förstått betydelsen av enzymers substratspecificiteter och att det på grund av dessa kan vara utmanande att förbättra innehållet av erukasyra i växtoljor. DGAT2 är ett enzym som katalyserar den sista sammansättningen av fröolja. Enzymer är stora molekyler tillverkade av underenheter som kallas aminosyror som sitter ihop i en lång kedja. Dessa aminosyror bestämmer beteendet hos enzymer, och därmed deras substratspecificitet. Genom att ändra några av aminosyrorna i DGAT2 har vi lyckats förändra enzymer som ogillar erukasyra till enzymer som gärna använder erukasyra. De detaljerade studierna av enzymer som är involverade i produktion av olja hjälper till att sträva efter bättre anpassade oljor.

Acknowledgements

This thesis is in no way a solo endeavour even though my name is the only one occurring on the front cover. I owe everybody in my surrounding my gratitude. My contribution to the scientific community summarised here would be impossible without them and all the efforts made by other researchers.

This work would not have existed at all without the kind support and encouragement from my group of supervisors, which consists of Ida Lager, Sten Styme, Anders S. Carlsson and Li-Hua Zhu. Ida, my main supervisor, thank you for believing in me when things were looking bleak and for supporting me through thick and thin. You went that extra mile for me and you will always be an inspiration to me. You have always encouraged me to develop my ideas, no matter how ridiculous they were. You have sincerely affected me, and have made me become a better person. My co-supervisor Sten is also a great source of inspiration, your genuine interest, kindness, knowledge, curiosity, critical thinking and your strong belief in cooperation are just a few of the characters, for which I admire you.

I am very grateful to have become a part of the close collaboration between our group and the professor Antoni Banaś and Kamil Demski, initiated by Sten and Antoni decades ago, and many of the findings in this thesis is a direct result of this collaboration. It has been an honour to work and spend time with Kamil, who is a brilliant aspiring young researcher and a fantastic friend, whom I've grown very fond of. Antoni has always been encouraging and very keen on welcoming us as guests and made us feel like a part of his family.

I would also like to thank my office mates Joel, Magnus and Per, as you endured my exaggerated exaltation every single time I believed that I had solved the mysteries of lipid metabolism and for rescuing me during my downfall, once I realised I was wrong. Per Snell is one of those, unique personalities, that I

nowadays recognise as one of my closest friends. Despite his fierce appearance, he is one of the kindest, supportive, generous persons I have ever met, who always do his uttermost for the collective well-being.

Thank you, Roland, for becoming my senpai long ago during our adventures somewhere in the arctic archipelago. With his excessively entertaining stories, he inadvertently (or deliberately, I'm quite unsure) convinced me to pursuit for an academic profession, and may very well be the reason for me becoming a doctoral student.

Rui, Meta and Per Hofvander thank you for being fantastic people that share my passion for old Studio Ghibli classics. I would also like to show my gratitude towards Helena, Helle, Helén and Mirela, four energetic individuals who spread joy and warmth in the lab and make me look forward to show up at work every time.

I would also like to show my greatest gratitude to the plant lipid research community. I have a hard time to believe that there is a more open-minded warm-hearted bunch of people within the academic world. I would especially like to thank Ed Cahoon, who has a beautiful mindset and Kent Chapman with his kind heart and generous personality. A wide range of beverages has been devoured, on numerous occasions and different continents, during endless discussions with Phil Bates whether the specificity of acyltransferases can provide a mechanism for the different acyl pools he so passionately advocates, or whether they exist at all. Further, his brothers in arms, Jay Shockey and Jay Thelén, both prominent researchers, treated me as an equal instantaneously and provided us with more pitchers than I would like to recall. That night, was also the night when our head of department persistently complained, that the Germans are unable to construct straight roads, while he was singing cheerfully. Thank you, Till Ischebeck, for encouraging your doctoral students not to fear authorities and include them in your companionship.

Finally, I would like to thank my closest family Annika, Abbe and Edith for all the encouragement, support and love during this whole process. Thank you, my closest relatives, Rolf, Winnie and Marie for being there when I needed you the most and for all the support, help and warmth.

Acta Universitatis agriculturae Sueciae
Doctoral Thesis No. 2020:24

Acyltransferases catalyse the acylation of glycerol into triacylglycerol, the main constituent in seed oil. The substrate specificities of acyltransferases, responsible for triacylglycerol synthesis, affect the fatty acid composition, which determines the properties of the seed oil. Here, we have identified a wide range of acyltransferases from several plant species and assessed their substrate specificities. Further, we have identified an amino acid region affecting the acyl donor specificity of an essential acyltransferase, acyl-CoA:diacylglycerol acyltransferase 2.

Simon Jeppson received his graduate education at the Department of Plant Breeding, SLU, Alnarp and obtained his MSc degree in Biology at SLU, Alnarp.

Acta Universitatis agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).

SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.

Online publication of thesis summary: https://pub.epsilon.slu.se

ISSN 1652-6880
ISBN (print version) 978-91-7760-566-9
ISBN (electronic version) 978-91-7760-567-6