Metabolic Engineering of *Crambe abyssinica* for Producing High Erucic Acid Oil

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Cover: crambe plants, flower and developing seeds, transciptome assembly, gene expression and sample correlation analysis (photos by Li-Hua Zhu and Danni Wang; Figures by Benjamin Almeida and Rui Guan)

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Abstract

Vegetable oils are renewable and more environmentally friendly than fossil oils, and thus are good alternatives to fossil oils for industrial applications. Erucic acid ($C22:1^{\Delta 13}$) is a valuable feedstock in the chemical industry. The current plant sources for erucic acid production are high erucic acid rapeseed (HEAR) and mustard. However, these crops readily outcross with food quality rapeseed, causing food oil safety concerns. *Crambe abyssinica* (crambe) is a dedicated non-food industrial oil crop, accumulating 22:1 to up to 60% of its seed oil, mainly in triacylglycerol (TAG). Further increasing the 22:1 content in crambe oil would reduce its downstream processing costs for purification or, if levels above 90% can be achieved, enable its direct application in the chemical industry.

This thesis focused on producing ultra-high erucic acid in crambe through genetic engineering. By modifying three genes (*BnFAE1, CaFAD2-RNAi* and *LdLPAAT*) involved in the biosynthesis of 22:1, genetically modified (GM) crambe lines that accumulate up to 73% of erucic acid in seed oil were developed. For re-transformation of the best erucic line to further increase the 22:1 level, a hygromycin based selection marker system was established. To study the phosphatidylcholine (PC) involvement in the accumulation of 22:1 in crambe, candidate genes encoding three enzymes (LPCAT, PDCT and PDAT) were cloned from wild type (WT) crambe. Several RNAi expression vectors with these genes were transformed into WT crambe. Significant changes in fatty acid composition were observed in the transgenic lines but not an increase in 22:1 level. The functions of these three enzymes in the 22:1 accumulation were discussed. By *in vitro* and *in vivo* investigation of enzymatic activities in GM and WT crambe, a bottleneck to higher 22:1 accumulating at early stages of seed development in GM crambe was pointed out. Comparative transcriptomic analysis of GM and WT crambe was carried out to identify key genes and to further elucidate the molecular mechanisms involved in 22:1 biosynthesis and TAG accumulation.

Keywords: Crambe abyssinica, erucic acid, genetic engineering, lipid biochemistry, fatty acid biosynthesis, triacylglycerol, phosphatidylcholine, RNAi, transcriptomics

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Dedication

To my parents, mentors and friends

These are the voyages of the starship Enterprise. Its continuing mission: to explore strange new worlds, to seek out new life and new civilizations, to boldly go where no one has gone before.

Star Trek: The Next Generation

Contents

List of Publications

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

I Li, X., van Loo, E.N., Gruber, J., Fan, J., Guan, R., Frentzen, M., Stymne, S. and Zhu, L.H. (2012). Development of ultra–high erucic acid oil in the industrial oil crop *Crambe abyssinica*. *Plant Biotechnology Journal*, 10(7), 862-870.

II Li, X., Fan, J., Gruber, J., Guan, R., Frentzen, M. and Zhu, L.H. (2013). Efficient selection and evaluation of transgenic lines of *Crambe abyssinica*. *Frontiers in Plant Science*, 4:162. DOI: 10.3389/fpls.2013.00162

III Guan, R., Li, X., Hofvander P., Zhou X.R., Wang, D.N., Stymne, S. and Zhu, L.H. RNAi targeting putative genes in phosphatidylcholine turnover results in significant change in fatty acid composition in *Crambe abyssinica* seed oil. *Lipids* (under revision).

IV Guan, R., Lager, I., Li, X., Stymne, S. and Zhu, L.H. (2014). Bottlenecks in erucic acid accumulation in genetically engineered ultrahigh erucic acid *Crambe abyssinica*. *Plant Biotechnology Journal*, 12(2), 193-203.

V Guan, R., Kushwaha S.K., Li, X., Almeida B., Hofvander P., Stymne, S. and Zhu, L.H. Comparative transcriptome analysis of *Crambe abyssinica* during the seed development focusing on lipid metabolism and erucic acid accumulation. (Manuscript).

Paper I, II and IV are reproduced with the kind permission of the publishers.

The contribution of Rui Guan to the Paper I-V included in this thesis was as follows:

I Participated in the selection of high erucic acid transgenic crambe lines by GC analysis and plant material management.

II Was involved in cloning of the internal reference gene and the gene expression analysis as well as plant material management.

III Planned and conducted the experiments and all data analysis. Wrote the paper together with the co-authors.

IV Planned and conducted all the experiments and data analysis. Wrote the paper together with the co-authors.

V Conceived, planned and conducted the study, analyzed data together with the bioinformaticians and wrote the manuscript with the co-authors.

Abbreviations

ACCase	acetyl-CoA carboxylase
acyl-CoA	acyl-Coenzyme A
ATP	adenosine triphosphate
CRISPR	Clustered Interspaced Short Palindromic Repeats
DAF	days after flowering
DAG	diacylglycerol
DGAT	acyl-CoA:diacylglycerol acyltransferase
FA	fatty acid
FAS	fatty acid synthase
FAD2	delta-12 fatty acid desaturase; omega-6 oleate desaturase
FAD3	delta-15 fatty acid desaturase; omega-3 linoleate desaturase
FAE	fatty acid elongase
GC	gas chromatography
GM	genetically modified
G-3-P	glycerol-3-phosphate
GPAT	acyl-CoA:glycerol-3-phosphate acyltransferase
HEAR	high erucic acid rapeseed
KCS	3-ketoacyl-CoA synthase
LEAR	low erucic acid rapeseed
LPA	lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferase
LPC	lysophophatidylcholine;1-acylglycerol-3-phosphocholine
LPCAT	acyl-CoA:lysophosphatidylcholine acyltransferase
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PDAT	phospholipid:diacylglycerol acyltransferase
PDCT	phosphatidylcholine: diacylglycerol cholinephosphotransferase

PTGS	post-transcriptional gene silencing
PUFA	polyunsaturated fatty acid
RACE	rapid amplification of cDNA ends
RNAi	RNA interference
TAG	triacylglycerol
TLC	thin layer chromatography
TF	transcription factor
TALEN	transcription activator-like effector nuclease
VLCFA	very long chain fatty acid
WRI1	WRINKLED 1
WT	wild type
8:0	caprylic acid
10:0	capric acid
12:0	lauric acid
16:0	palmitic acid
18:0	stearic acid
18:1	$C18:1^{\Delta 9}$; oleic acid
18:1-CoA	oleoyl-CoA
18:2	linoleic acid; LA
18:3	alpha-linolenic acid; ALA
22:1	$C22:1^{\Delta 13}$; erucic acid
22:1-CoA	erucoyl-CoA

1 Purposes

The ultimate goal of this work is to significantly increase the erucic acid level in seed oil of the dedicated industrial oil crop *Crambe abyssinica* through genetic engineering and molecular and biochemical controls of seed oil biosynthesis. The thesis work belongs partially to the EU-ICON project (Industrial Crops producing added value Oils for Novel chemicals), a large international research project supported by the European Commission 7th Framework Program for Research and Technological Development.

The overall goals of this thesis are to:

1) Gain a better understanding of molecular and biochemical regulations of seed oil biosynthesis in *C. abyssinica*, with particular focus on the accumulation of erucic acid in seed oil.
2) Demonstrate how the gene technology could be used for speeding up the plant breeding process and increasing possibilities for genetic enrichments.

The specific purposes of this thesis are to:

1) Increase the erucic acid content to an unprecedented level in the seed oil.
2) Elucidate the limiting factors affecting erucic acid accumulation.
3) Conduct a functional analysis of newly identified candidate genes for their involvements in the erucic acid biosynthesis.
4) Identify new candidate genes which are essential for erucic acid accumulation.

2 Introduction

2.1 Plant oils and their industrial applications

Oils in plants are important metabolites with the highest energy density of different carbon reserves (Bates *et al.*, 2013). The most common form of plant oils is triacylglycerol (TAG), in which three fatty acid (FA) chains are esterified to the glycerol backbone. The oil quality is determined by the composition of the FA chains, which varies in the carbon chain length, degree of saturation and unusual moieties (Dyer *et al.*, 2008). Plant oils are used mainly for food and feed, as a major supplier of calories and essential FAs. However, due to their special properties, plant oils can also be used as renewable feedstocks in the chemical industry for value-added inedible applications such as cosmetics, paint/varnish, alkyd resins, plastics and lubricants (Dyer *et al.*, 2008).

Global production of vegetable oils is estimated to have reached over 169 million metric tonnes (Mt) during 2013 to 2014 (USDA, 2014). The major supply of plant oils comes from palm (36%), soybean (26%), rapeseed (15%), sunflower seed (9%) and palm kernel (4%). Peanut, cottonseed, coconut and other oil crops account for 10% of global vegetable oil production (Figure 1). Nowadays more than 70% of the plant oil produced is used for food and nutritional purposes but 20% goes to the chemical industry, as the price is becoming competitive with that of fossil oils (Carlsson *et al.*, 2011). An increasing proportion of plant oil is also being used for biodiesel production which now constitutes about 12% of global plant oil production (REN 21, 2014).

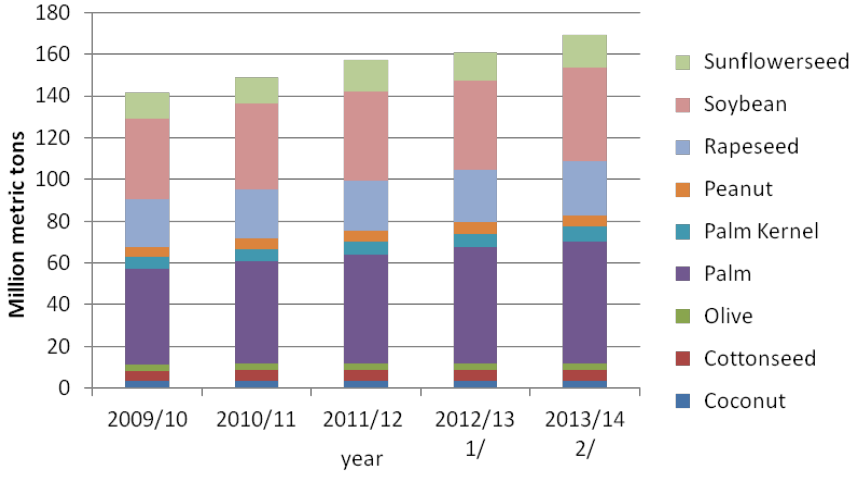

Figure 1. World vegetable oil supply and distribution, 2009/10-2013/14. 1/ Preliminary. 2/ Forecast. (data from USDA, 2014)

2.2 Replacing fossil fuel with plant oils in the chemical industry

The world demand for fossil oils is estimated to be 93.8 million barrels per day (mb/d) by 2015, while the world supply was down to 92.9 mb/d in August 2014, according to the latest report from the International Energy Agency (IEA, 2014). New exploration of unconventional crude oil resources such as shale oil might mitigate the temporary crisis in oil supply, but the future perspective is quite uncertain considering that *e.g.* shale oil processing could pose threats to water safety (Vidic *et al.*, 2013). Even though the crude oil price can decrease temporarily as observed in the third quarter of 2014, in the long-term there will be a fossil oils peak and depletion afterwards, thus making the searching for renewable alternatives extremely important (Chapman, 2014).

The fossil fuel reservoir is mainly the carbon reserve from ancient autotrophes such as plankton and algae. The transformation happened billions of years ago and has changed the carbon-based plant and animal remains into the liquid hydrocarbons that move and migrate, ultimately becoming the oil and gas reservoirs found today (Kenney *et al.*, 2002). In contrast, plant oils are 'fresh' oils, thus making them a logical substitute for fossil oils. Increasing use of plant oils as liquid fuel has been observed in Europe, *e.g.* biodiesel derived from rapeseed, sunflower seed and palm oils (Durrett *et al.*, 2008).

As the global production of plant oils is equivalent to only 3% of the world consumption of fossil oils, it is currently not realistic to replace the major source of fuel with plant oils. However, only about 10% of crude oils are used

in the chemical industry annually, and it is thus possible to replace a significant proportion of fossil oils with plant oils in this case. The value of the products made from this 10% of fossil oil is almost equal to the value of the fuels made from the rest. Moreover, refinery processing of crude oils to extract the compounds required in the chemical industry is highly costly, energy-consuming and environmentally unfriendly. It is thus beneficial from both an economic and environmental point of view to replace the fossil oils in the chemical industry with plant oils.

Plant oils naturally provide a wide range of unique oil qualities due to their particular FA compositions (Aitzetmüller *et al.*, 2003), making it possible to reduce the cost of downstream processing and lower the price of the end product(s). Minor oil crops or wild species predominantly accumulate unusual FAs with outstanding industrial values. Some of these unusual FAs are:

1) Medium-chain FAs, *e.g*. caprylic (C8:0) and capric acid (C10:0) in *Cuphea* oils and lauric acid (C12:0) in palm kernel and coconut oils;

2) Hydroxy FAs, *e.g*. ricinoleic acid (12-hydroxy C18:1$^{\Delta 9}$) in castor bean oil;

3) Conjugated FAs, *e.g*. α-eleostearic acid (C18:3$^{\Delta 9c,11t,13t}$) in tung tree oil;

4) Very-long-chain FAs, *e.g*. erucic acid, C22:1$^{\Delta 13}$ in crambe seed oil (Dyer *et al.*, 2008; Vanhercke *et al.*, 2013).

The question is whether it is currently realistic to replace all fossil oils used in the chemical industry with plant oils. Our vision is to replace 40% of the fossil oils in petrochemical application with vegetable oils and this will require global production of plant oils to treble in the next two decades (Carlsson *et al.*, 2011). However, it will be very difficult to achieve this trebling with the existing oil crops, as their oil levels and yields have reached a plateau. Another obstacle is that the FA composition of natural plant oils does not always meet end-use requirements. Many oil plants accumulating unusual FAs are not domesticated or possess unfavorable agricultural traits, such as low seed yield, or contain toxic or allergenic compounds (Vanhercke *et al.*, 2013).

The development of new and highly efficient industrial oil crop platforms would be one way to deal with the above mentioned challenges. It should be borne in mind that cultivating oil crops for industrial purposes is not intended to compete with food crops to secure the world food supply. The increase in plant oil production should therefore be achieved by increasing the oil yield per hectare or plant oil content without increasing the use of agricultural land and by developing new oil crops that can utilize the marginal lands that are not used for food production.

2.3 Methods to optimize plant oil quality

Using the natural variation present in the different genotypes, conventional breeding methods have been applied to some oil crops to increase the oil content and alter the oil quality. For example, plant oils with a high content of oleic acid (C18:$1^{\Delta 9}$) are preferred for certain food and chemical applications due to their oxidative stability at high temperature. Safflower (*Carthamus tinctorius*) oils rich in linoleic acid (C18:$2^{\Delta 9,12}$) are widely used as industrial oils *e.g*. in surface coating. For food applications such as frying oil, safflower hybrid varieties with optimized proportions of oleic acid and linoleic acid have been obtained by crossing and backcrossing to the high oleic acid safflower variety (Knowles, 1969). In a recent study on the seed oils of *Guizotia abyssinica*, which predominantly accumulates linoleic acid, a large variation in the oleic acid content was found in the populations. The high oleic acid line provides a promising resource to develop stable lines with even higher content of oleic acid by crossing to the inbred lines and selection breeding (Geleta *et al.*, 2011).

In the oil crops lacking natural variation, induced mutation breeding methods have been conducted to improve the oil quality. Successes have been achieved in the development of sunflower oil with a high content of oleic acid (Pérez-Vich *et al.*, 2002) or a high content of stearic acid (C18:0) (Osorio *et al.*, 1995), and of soybean oil with a high content of stearic acid (Graef *et al.*, 1985), or low content of palmitic acid (C16:0) (Fehr *et al.*, 1991).

Modern breeding methods such as genetic engineering have greatly advanced breeding of oil plants and a number of new breeding lines or varieties with desired oil quality or quantity have been developed. For instance, post-transcriptional gene silencing (PTGS) was applied in the development of high stearic and oleic rapeseed (*Brassica napus*), high oleic mustard (*B. juncea*), high oleic and stearic cottonseed (*Gossypium hirsutum*) oils (Knutzon *et al.*, 1992; Stoutjesdijk *et al.*, 2000; Liu *et al.*, 2002). The recent development of super-high oleic safflower by CSIRO through the RNAi-mediated gene silencing strategy is regarded as a landmark revealing the great potential for metabolic engineering of oil plants to obtain oleochemicals with high purity (Vanhercke *et al.*, 2013). Newly emerging site-directed-mutagenesis techniques such as transcription activator-like effector nucleases (TALENs) have been applied to soybean to change the seed oil FA composition (Gaj *et al.*, 2013; Haun *et al.*, 2014).

3 Background

3.1 Erucic acid (22:1) and *Crambe abyssinica*

3.1.1 Applications and major sources of 22:1

There has been an increasing demand for erucic acid ($C22:1^{\Delta13}$) as feedstock in the chemical industry. Worldwide consumption of erucic acid is estimated to have almost doubled between 1990 and 2010, from 18 to 35 million tonnes (Dyer *et al.*, 2008). The main derivative of erucic acid is erucamide which is used in the plastic industry as an anti-block and slip agent and corrosion inhibitor for extruding polyethylene and propylene films to make commercial plastic bags. Other erucic acid-based applications include nylon, coatings, textile softeners, surfactants and lubricants (Leonard, 1993; Friedt and Luhs, 1998; Piazza and Foglia, 2001). Chemical modification of the double bond of $C22:1^{\Delta13}$ by ozone oxidation could generate pelargonic acid (C9 FA) and brassylic acid (a dicarboxy C13 FA), which are used in the perfume industry (Töpfer *et al.*, 1995). In a pharmaceutical application, erucic acid is combined with oleic acid to treat X-linked adrenoleukodystrophy (ALD), a type of peroxisomal diseases and genetic disorder (Rizzo *et al.*, 1989).

Erucic acid belongs to the very-long-chain FAs (VLCFAs) group with carbon numbers above 18. In plants, VLCFAs and their derivatives are precursors or components of many important metabolites synthesized in different cell types, such as sphingolipids, suberin, cuticular waxes and pollen coat (Haslam and Kunst, 2013). There are abundant sources featuring with the accumulation of erucic acid and other VLCFAs in nature. As the word 'erucic' referred to *Eruca*, a genus of Brassicaceae family, erucic acid and other VLCFAs are commonly associated with plants from the Brassicales order. As shown in Table 1, plants predominantly accumulating erucic acid or other VLCFAs are found in the Brassicaceae and its genera such as *Brassica*, *Crambe* and *Sinapsis*; in the Tropeaolaceae species such as nasturtium

(*Tropaeolum majus*) and in the Limnanthaceae species such as meadowfoam (*Limnanthes douglasii*) (Mikolajczak *et al.*, 1961; Ackman, 1990; Löhden and Frentzen, 1992; Leonard, 1993; Lazzeri *et al.*, 1997).

Table 1. *Species of Brassicales accumulating erucic acid (C22:1) or other VLCFAs in the seed oil*

Family	Genus	Species/Sub-species	major VLCFAs and percentage
Brassicaceae	*Brassica*	*Brassica napus*	C22:1 (45-50%)
	Crambe	*Crambe abyssinica*	C22:1 (56-60%)
	Sinapsis	*Sinapsis alba*	C22:1 (33%)
Tropeaolaceae	*Tropaeolum*	*Tropaeolum majus*	C22:1 (65-78%), C20:1 (17-25%)
Limnanthaceae	*Limnanthes*	*Limnanthes douglasii*	C20:1 (63%), C22:1 (14%), C22:2 (12%)

At present, the major plant sources of C22:1$^{\Delta 13}$ for commercial production are high erucic acid rapeseed (HEAR) and mustard. HEAR accumulates 45-50% of erucic acid in the seed oil, while the mustard oils extracted from *S. alba* and *B. juncea* contain 26-33% erucic acid (Issariyakul *et al.*, 2011). However, the polyunsaturated FAs (PUFAs) still account for quite a high proportion in the HEAR and mustard oils, and it is expensive to separate these PUFAs from erucic acid in downstream processing in the chemical industry. Economically viable application of HEAR requires its erucic acid content to be further increased. In a study using asymmetric somatic hybridization, a moderately high content of erucic acid (51%) was obtained in HEAR (Wang *et al.*, 2003). A more drastic increase in erucic acid (up to 72%) was achieved by cross-breeding in combination with genetic engineering of HEAR (Nath *et al.*, 2009).

However, there is a major food safety concern with oils containing high erucic acid content, as an early animal study suggested that consumption of large amounts of erucic acid may result in cardiovascular problems (Beare-Rogers *et al.,* 1974). To eliminate erucic acid in food oils, decades of efforts have been devoted to developing varieties for food applications, such as canola, a low erucic acid rapeseed (LEAR) developed in Canada (Ackman, 1990). As the potential out-crossing of HEAR and LEAR may cause a potential increase in erucic acid in the food chain, the cultivation of HEAR has been constrained. As seen in the U.S.A almost two decades ago, the expansion of canola cultivation area has resulted in a drastic decrease in HEAR cultivation (Li *et al.*, 2012). Moreover, the application of genetic engineering in the development of HEAR makes it more difficult to commercialize due to the food security concerns under the current strict legislation on genetically modified (GM)

crops in the EU. Thus for industry oil production, it is clearly necessary to find other oil crops that do not outcross with existing food oil crops.

3.1.2 *Crambe abyssinica* as an alternative source of 22:1

Crambe abyssinica (shown in Figure 2), commonly known as abyssinica kale, abyssincia mustard or crambe, is an annual species originated in Ethiopia and East Africa (Leppik and White, 1975). The mature crambe seeds accumulate fairly high amounts of oil (ca. 35-38% with pod and up to 50% without pod) and protein (26% with pod).

Figure 2. Crambe (Crambe abyssinica) plants in the field (left), crambe flowers (centre) and developing crambe seeds (right) (photos by Li-Hua Zhu and Danni Wang).

In crambe, erucic acid (22:1) accounts for 55-60% of the total seed oil (Gurr *et al.*, 1972; Gurr *et al.*, 1974; Appleby *et al.*, 1974), thus it is of outmost interest to be used as an industrial crop (Lazzeri *et al.*, 1994; Carlsson, 2009). Crambe is reported to be a hexaploid (2n=6X=90) (Warwick and Gugel, 2003; Aitzetmüller *et al.*, 2003). The most closely related species but distinguished by the chromosome number are *C. hispanica* (2n=4X=60) and *C. glabrata* (2n=2X=30) (Warwick and Gugel, 2003). *Crambe abyssinica* is the only cultivated species in the genus (Leppik and White, 1975) and was recorded to be first cultivated in former USSR and later introduced to USA and Canada (Oplinger *et al.*, 1991). Nowadays the cultivation of crambe is scattered in central and north Europe and Asia, where it is mainly grown as a spring annual crop or winter annual crop in the regions with mild winters. It is now also cultivated in Brazil where it has been found to be resistant to nematodes and tolerant to dry conditions (Carlsson *et al.*, 2014).

The advantages of using crambe as an alternative source of erucic acid include: 1) it contains the highest level of erucic acid in the seed oil naturally occurring within the Brassicaceae, 10-15% higher than the current HEAR oils; and 2) crambe is essentially self-pollinated and does not out-cross with any other food oilseed crops or closely related wild species (Fitzjohn *et al.*, 2007).

However, the seed and oil yields per hectare of crambe are lower than that of HEAR (Wang *et al.*, 2004) and the seed cake cannot be used in feed due to high amounts of glucosinolates. To make crambe economically competitive with HEAR, its erucic acid content needs to be further increased without a negative impact on the total oil content.

Conventional selection breeding for improving oil quality is not effective in crambe as the species lacks genetic variation for important agronomic traits including erucic acid and total oil content (Warwick and Gugel, 2003). Modern breeding methods, such as genetic engineering, offer great possibilities for oil improvement and erucic acid accumulation in crambe through broadening the gene pools. Significant progress has been made in crambe by genetic engineering to produce industrial oils in the recent years (Li *et al.*, 2010; Li *et al.*, 2012).

3.2 Metabolic pathways of seed oil biosynthesis

The complex metabolic process of seed oil biosynthesis essentially associates with the carbon fixation via photosynthesis, with carbon partitioning towards more hydrocarbons to form storage lipids. Triacylglycerol (TAG) is the major form of seed oil. The formation of TAG molecules involves the supply of FAs (in the form of acyl-Coenzyme As, acyl-CoAs) as building blocks and their assembly to the glycerol backbone (in the form of glycerol-3-phosphate, G-3-P). In general, previous studies on FA synthesis and TAG assembly in oilseeds have focused on source of the substrates, the rate-limiting enzymes or major regulatory factors, the equilibrium of the enzymatic reactions and the compartmentation of multiple pathways at subcellular level.

3.2.1 *De novo* fatty acid synthesis

Unlike in other eukaryotic organisms, *de novo* FA synthesis in plants occurs in plastid stroma rather than in the cytosol (Ohlrogge and Jaworski, 1997).

The major committed carbon source in seed for FA synthesis is plastidial acetyl-CoA, which is regarded as the hub metabolite for different carbon reserves (Oliver *et al.*, 2009). In Arabidopsis seeds, the plastidial pyruvate dehydrogenase complex (PDH) has been shown to be the enzyme directly catalyzing pyruvate to generate acetyl-CoA, NADH and CO_2 (Johnston *et al.*, 1997; Ke *et al.*, 2000). The production of pyruvate can be traced back to the sucrose metabolism. Hexose phosphates or triose phosphates produced via glycolysis in the cytosol can be transferred and utilized in the plastid to form pyruvate. Meanwhile, there is also a bypass glycolytic reaction via ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) in the developing

chlorophyll-containing oil seeds (Chapman and Ohlrogge, 2012). By reutilizing the CO_2 released by PDH, the pentose phosphate pathway (PPP) via RuBisCo can increase the acetyl-CoA production by 20% (Schwender *et al.*, 2004).

The first priming reaction for FA synthesis is the formation of malonyl-CoA, generated from acetyl-CoA and bicarbonate catalyzed by the plastidial acetyl-CoA carboxylase (ACCase). ACCase is regarded as the rate-limiting enzyme for FA synthesis in plants (Nikolau *et al.*, 2003). There are two forms of ACCase in the dicots and most of the monocots: 1) the multifunctional homodimeric form which is localized in the cytosol; and 2) the heterometric form with four easily dissociated subunits in the plastid, among which the beta subunit of carboxyltransferase (β-CT) is the only plastome-encoded component (Sasaki *et al.*, 1995). However, in Gramineae species there are only two types of homodimeric isoforms localized in the plastid (herbicide-sensitive) and cytosol (herbicide-resistant), respectively (Konishi *et al.*, 1996).

Fatty acid synthase (FAS) in plants is a dissociable multi subunit complex. The malonyl moiety is transferred to acyl carrier protein (ACP) by malonyl-CoA:ACP malonyltransderase (MCMT) before being utilized by FAS. The condensation of malonyl-acyl carrier protein (ACP) and acetyl-CoA to form 3-ketoacyl-ACP is catalyzed by ketoacyl-ACP synthase III (KASIII), followed by reduction by ketoacyl-ACP reductase (KAR), dehydration by hydroxyacyl-ACP dehydrase (HAD) and another reduction by enoyl-ACP reductase (ENR) to form a 3-ketobutyrl-ACP (four-carbon unit). Further sequential condensation of two-carbon units and elongation up to 16:0-ACP requires KASI and malonyl-CoA. After 16:0-ACP is formed, it can be elongated to 18:0-ACP by KASII and further desaturated to $18:1^{\Delta 9}$-ACP by delta-9 stearoyl-ACP desaturase (SAD).

Both 16:0-ACP and $18:1^{\Delta 9}$-ACP can enter so called prokaryotic glycerolipid pathway within the plastid, but in oil seeds nearly all the acyl groups will undergo further modification along the eukaryotic pathway in the endoplasmic reticulum (ER) (Li-Beisson *et al.*, 2013). The acyl-ACP thioesterases of FATB and FATA types specific for 16:0-ACP and $18:1^{\Delta 9}$-ACP, respectively, can catalyze the production of free FAs. These FAs are then exported through the plastid envelopes and ligated to CoA by long-chain acyl-CoA synthetase (LACS) in the outer envelope membrane, where they are released as acyl-CoAs in the cytosol. The relative proportions of 16:0, 18:0 and $18:1^{\Delta 9}$ exported are decided by the activities of four enzymes: FATA, FATB, SAD and KASII. The FA chain length and degrees of saturation in the seeds can be altered by genetic engineering of these four enzymes (Cahoon *et al.*,

2010). The major FAs exported from plastid are 16:0 and 18:1 with usually minor amounts of 18:0.

Fatty acid synthesis is under the complex regulation of many factors (Marchive *et al.*, 2014). The activities of ACCase and FAS require ATP and reductants like NAD(P)H, and are thus under the regulation of photo system, phosphorylation and redox status. Protein interaction has been found between PII/AtGLB1 and the subunits of ACCase in Arabidopsis (Bourrellier *et al.*, 2010). Moreover, product feedback control of 18:1-ACP on ACCase has recently been reported (Andre *et al.*, 2012). The key transcriptional activators to the embryo development like LEAFY COTYLEDON 1 (LEC1) and LEC2 also regulate the FA synthesis (Andrianov *et al.*, 2010; Shen *et al.*, 2010). It is notable that a transcription factor (TF) *WRINKLED 1* (*WRI1*) can directly co-regulate genes involved in the FA synthesis and in sucrose metabolism (Baud *et al.*, 2010).

3.2.2 Very long chain fatty acid (VLCFA) synthesis

The biosynthesis of VLCFAs takes place outside the plastid, catalyzed by enzymes bound in the ER that are quite analogous to the FAS in the plastid, but utilizes different initial precursors and different sources of two-carbon units. After *de novo* synthesis and export from the plastid, oleoyl-CoA (18:1-CoA) is believed to be the direct precursor to form C22:1, but studies in *B. napus* and *B. rapa* have showed there may be another intermediate pool contributing to this process (Hlousek-Radojcic *et al.*, 1995; Bao *et al.*, 1998). The elongation of C18:1 involves cytosolic malonyl-CoA as the two-carbon donor. Mitochondrial citrate formed in the tricarboxylic acid (TCA) cycle and transferred to the cytosol may be the precursor to produce this cytosolic acetyl-CoA (Oliver *et al.*, 2009), which is subsequently converted to malonyl-CoA by cytosolic ACCase for FA elongation (Bao *et al.*, 1998).

The elongation reaction of FA in the cytosol is initiated by the condensation reaction catalyzed by 3-ketoacyl-CoA synthase (KCS or FAE1), which is regarded as the essential enzyme for the activity of elongation complex and the decarboxylation of malonyl-CoA (Millar and Kunst, 1997; Blacklock *et al.*, 2006). Condensation is followed by reduction by a beta-ketoacyl-CoA reductase (KCR1), dehydration by a beta-hydroxyacyl-CoA dehydratase (HCD or PAS2) and another reduction by an enoyl-CoA reductase (ECR or CER10) (Haslam and Kunst, 2013).

The two-step elongation to form C22:1 consists of C18:1 being elongated to C20:1 and C20:1 being elongated to C22:1(Pollard and Stumpf, 1980; Agrawal and Stumpf, 1985). These two steps are catalyzed by the same enzyme, but the effectiveness in the second step may differ in plants accumulating mainly 20:1

as the VLCFA in the seed (*e.g.* Arabidopsis) and those accumulating 22:1 as the major VLCFA in TAG (*e.g.* crambe).

3.2.3 Triacylglycerol (TAG) assembly

Alternative pathways or combinations of pathways are used for TAG assembly in different plants. The stepwise acylation of the *sn*-1 and *sn*-2 position of G-3-P and one-step dephosphorylation to form diacylglycerol (DAG) is known as the Kennedy pathway (Kennedy, 1961). The biosynthesis of TAG shares a similar pathway with membrane lipids in the early steps where DAG is a common precursor.

De novo DAG assembly occurs at the ER membrane. The acyl acceptor, glycerol-3-phosphate (G-3-P) is believed to be formed in the cytosol from dihydroxyacetone phosphate (DHAP) catalyzed by NAD-dependent glycerol-3-phosphate dehydrogenase (GPDH). The first step of acylation is the formation of lysophosphatidic acid (LPA) from G-3-P catalyzed by *sn*-1 glycerol-3-phosphate acyltransferase (GPAT). Eight members of the GPAT gene family have been found in Arabidopsis, but none of these has been confirmed to be associated with membrane lipid or TAG synthesis (Li-Beisson *et al.*, 2013). Based on the sequence similarity, an ER-localized GPAT9 homologue to mammalian and yeast GPAT protein was speculated to be involved in the TAG assembly, but this needs further investigation (Gidda *et al.*, 2009).

Lysophosphatidic acid acyltransferase (LPAAT), catalyzing the acylation of LPA to form phosphatidic acid (PA), is the second step in TAG assembly. Given the complex expression patterns, several genes encoding LPAAT have been identified in Arabidopsis and other species. Some of these genes have been suggested to be essential for the plant development (Kim and Huang, 2004). Notably in the coconut endosperm and *Limnanthes* genus, LPAAT with specificity to C12:0 and C22:1, respectively results in the presence of these unusual FAs in the *sn*-2 position of TAG in their seed oils (Knutzon *et al.*, 1995; Knutzon *et al.*, 1999; Löhden and Frentzen, 1992). The last step of DAG formation is catalyzed by Mg^{2+}-dependent phosphatidic acid phosphatase (PAP) (Carman and Han, 2006).

The acyl-CoA dependent TAG biosynthesis from DAG is catalyzed by diacylglycerol acyltransferase (DGAT). Several DGATs have been found in Arabidopsis and other species, but so far only DGAT1 and DGAT2 have been shown to be related to the TAG biosynthesis (Lardizabal *et al.*, 2001). DGAT1 has been proven to be involved in the TAG accumulation in plants, both in oil seeds and vegetative tissues (Routaboul *et al.*, 1999; Bouvier-Navé *et al.*, 2000). DGAT2 with preference to ricinoleoyl-DAG has been suggested to be

associated with the dominant accumulation of ricinoleic acid (12-hydroxy C18:1$^{\Delta 9}$) in castor bean oil (He *et al.*, 2004). In tung tree accumulating conjugated FA (C18:3$^{\Delta 9c,11t,13t}$) in oil, *DGAT1* and *DGAT2* have been found with non-redundant function in TAG synthesis, with the *DGAT2* reported to be highly expressed in the developing seeds (Shockey *et al.*, 2006). The contribution of *DGAT1* and *DGAT2* to the TAG accumulation may vary among different plant species and tissues. In addition, disruption of the *DGAT1* expression in Arabidopsis has resulted in only a 20% to 40% decrease in TAG in seeds, indicating that other genes are also involved in TAG biosynthesis (Katavic *et al.*, 1995).

3.2.4 Phosphatidylcholine (PC) involvement in polyunsaturated fatty acid and TAG biosynthesis

Phosphatidylcholine (PC) is the substrate for the production of linoleate (18:2) from oleoyl-PC by the delta-12 FA desaturase (FAD2) enzyme, but also for the further delta-15 desaturation of 18:2-PC to linolenoyl (18:3)-PC by the FAD3 enzyme (Stymne and Appelqvist, 1978; Arondel *et al.*, 1992; Okuley *et al.*, 1994). Virtually all polyunsaturated fatty acids (PUFAs) in TAG are synthesized on PC. In a recent study on Arabidopsis, PC has been indicated to be an immediate acceptor for oleoyl-CoA (18:1-CoA) after its formation in the plastid in Arabidopsis (Bates and Browse, 2011).

PC metabolism is also involved in the acyl editing by the forward and reverse reaction of 1-acylglycerol-3-phosphocholine (LPC) acyltransferase (LPCAT) (Stymne and Stobart, 1984; Lager *et al.*, 2013). This enzyme has also been suggested to be involved in transferring acyl groups between plastid and ER membrane. Modified acyl groups esterified to PC can enter the acyl-CoA pool for the Kennedy pathway by the reverse reaction of LPCAT. The Arabidopsis double-mutant of *lpcat1/lpcat2* has been shown to increase VLCFA and decrease PUFA in the seed oil (Wang *et al.*, 2012).

Acyl-CoA independent TAG formation involves PC as the acyl donor to the *sn*-3 position of DAG, catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT) in yeast and plants (Dahlqvist *et al.*, 2000). PDAT1 and DGAT1 have been indicated to have an overlapping function in seed oil accumulation, and the function of one of these enzymes is essential to the normal development of seeds and pollen in Arabidopsis (Zhang *et al.*, 2009).

PC:DAG cholinephosphotransferase (PDCT) encoded by the *ROD1* gene in Arabidopsis has been identified as being associated with the PC-DAG inter-conversion and has been shown to be important for PUFA accumulation in TAG. Disrupted expression of the *ROD1* gene has resulted in a 40% decrease in PUFA content in Arabidopsis seed oil, without any impact on total TAG

accumulation (Lu *et al.*, 2009). Furthermore, a drastic reduction in PUFA content, of 66%, has been shown in the Arabidopsis triple-mutant *lpcat1/lpcat2/rod1* (Bates *et al.*, 2012). These studies reveal the importance of PC-involved pathways and their combined contributions to the PUFA and TAG biosynthesis.

3.3 Genetic engineering of the oil quality

Due to the complexity of the lipid metabolism, there are still questions and unidentified regulation mechanisms for TAG biosynthesis in oilseed plants. Genetic engineering in the model species Arabidopsis has generated numerous materials for investigating the TAG metabolism in oilseeds, and has helped to develop novel oil crops with specific quality for potential applications.

By silencing the endogenous genes and/or introducing exogenous genes encoding the essential enzymes, the FA composition in seed oils can be altered regarding degrees of unsaturation, chain length and other modifications such as hydroxy-, epoxy-, acetylenic- and cyclopropane groups in the acyl chains, *etc*. Some examples are shown here.

1) Oleic acid: by silencing the oleate-PC delta-12 FAD2, oleic acid has been drastically increased in the seed oils of rapeseed, cottonseed and safflower, etc. as mentioned in the Introduction chapter of this thesis.
2) PUFA: alpha-linolenate (C18:3) has been substantially increased to 70.9% in soybean seed oil by expressing a bifunctional delta-12 and delta-15 FAD from fungi (Damude *et al.*, 2006).
3) Medium-chain FAs: caprylic (C8:0), capric acid (C10:0) and lauric acid (C12:0) have been produced up to 7 mol %, 29 mol % and 63 mol % respectively in rapeseed by expressing different medium chain acyl-ACP thioesterases (Wiberg *et al.*, 2000).
4) Acetylenic and epoxidated FAs at levels up to 25% and 15% respectively, have been produced in Arabidopsis seed oil by expressing divergent FAD2-like enzymes (Lee *et al.*, 1998).
5) Hydroxy FAs: ricinoleic acid (12-hydroxy C18:1$^{\Delta 9}$) has been produced at levels of nearly 30% in Arabidopsis seed oil by co-expressing the castor bean genes encoding a delta-12 hydroxylase (RcFAH12), a PDAT (RcPDAT1A) and a DGAT (RcDGAT2) (Van Erp *et al.*, 2011).
6) Cyclopropene FAs have been reported to accumulate up to 35% in the oil of individual T_1 seeds in Arabidopsis, by co-expressing a *Escherichia coli* cyclopropane synthase gene and a gene encoding LPAAT from *Sterculia foetida* (Yu *et al.*, 2014)

The success of genetic engineering of oil crops for certain oil quality relies mainly on: 1) A comprehensive understanding of the biosynthesis of certain FAs and identification of important genes involved in the related metabolic pathways; and 2) establishment of effective and stable delivery systems for transgene modules. In addition, the transformation protocols have been established in many oil crops, such as camelina, safflower and crambe *etc.*, providing abundant platforms for optimizing oil quality (Carlsson *et al.*, 2014).

4 Genetic engineering of crambe for high 22:1 content

4.1 Research strategy and design

The research strategies used in this thesis for increasing 22:1 content in crambe include:

1) Increasing the proportion of 22:1 incorporation at the *sn*-2 position of TAG.
2) Reducing conversion from 18:1 to 18:2.
3) Reducing the 18:1 flow to PC.

Although crambe can naturally accumulate high amounts of 22:1 (up to 60%) in the seed oil, the extremely low activity of endogenous LPAAT enzyme in crambe to incorporate 22:1-CoA at the *sn*-2 position of TAG limits the maximum accumulation of 22:1 to 66% in theory. This is why erucoyl-CoA accounts for 86% of the FAs at *sn*-1+ *sn*-3 positions, while only 8% at the *sn*-2 position are reported to be 22:1-CoA in the seed oil of WT crambe (Li *et al.*, 2012). Therefore, introduction of an LPAAT enzyme from another species that can effectively incorporate 22:1 at the *sn*-2 position seems to be one major way to increase 22:1 content in crambe. Meanwhile, over 20% of the FAs in the TAG of WT crambe are PUFAs (18:2 and 18:3), indicating the potential for increasing the proportion of 22:1-CoA through blocking the conversion of 18:1 to 18:2 and further to 18:3 (Li *et al.*, 2012).

The abovementioned strategies were employed in different papers included in this thesis through essentially genetic transformation using a method described previously (Li *et al.*, 2010; Li *et al.*, 2011). The structure of all the papers included in this thesis is summarized in Figure 3.

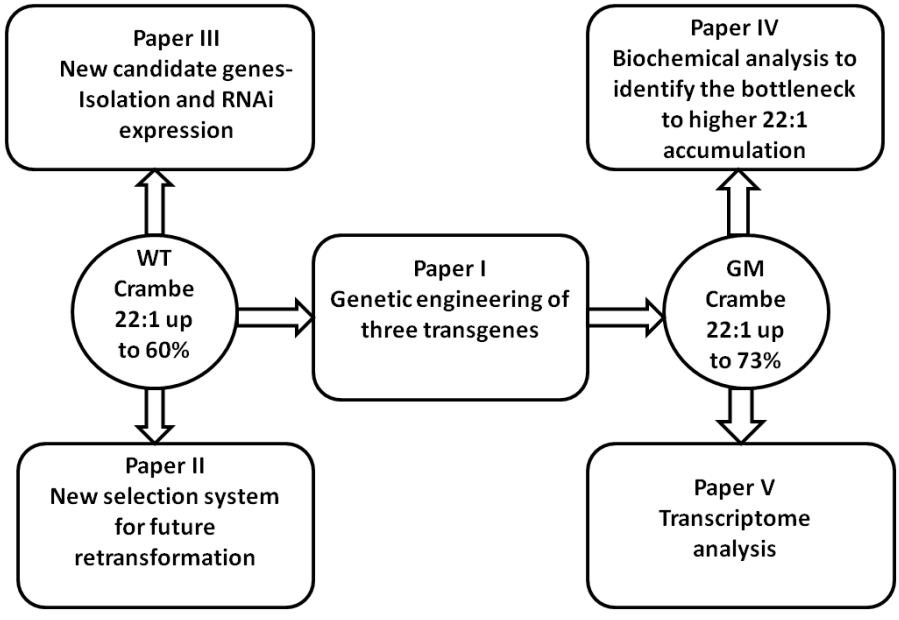

Figure 3. Structure and interrelationships between the studies reported in Paper I-V of this thesis. WT, wild type; GM, genetically modified.

In Paper I, transgenic crambe lines harboring three target transgenes were generated, as indicated in Figure 4. These target genes were:

i) *LdLPAAT* from *Limnanthes douglasii* for incorporation of 22:1-CoA at the *sn*-2 position of TAG;

ii) *BnFAE1* from *Brassica napus* for promoting the VLCFA biosynthesis to form 22:1;

iii) *CaFAD2-RNAi* from *C. abyssinica* for reducing the conversion from 18:1 to 18:2, thus increasing the 18:1 pool for the 22:1 synthesis.

Since the GM crambe line with 22:1 content up to 73% obtained in Paper I was transformed with the *nptII* gene as the selective marker, a new selection system for retransformation of this line was required for further increasing the 22:1 content. For this purpose, a hygromycin-based selection system was established in crambe for future retransformation of the GM lines for ultra-high 22:1 content and/or other desirable traits in Paper II**.** To eliminate the 18:1 flow to PC or to ensure the 18:1 pool for 22:1 synthesis, PC involvement in 22:1 accumulation was investigated in Paper III**.** In that paper, three candidate genes encoding enzymes LPCAT, PDCT and PDAT were isolated from WT crambe. Several RNAi expression vectors with these genes were transformed into WT crambe and transgenic lines were evaluated.

In Paper IV, comparative biochemical analysis of WT and GM crambe was conducted to elaborate the mechanism of 22:1 accumulation and the distribution of 22:1 during the seed development.

Several questions and speculations about the bottleneck to accumulating even higher 22:1 were raised in Papers III and IV, indicating a need for more comprehensive studies. Therefore, comparative transcriptomic study of crambe was carried out in Paper V using the developing seeds of WT and GM crambe with the focus on the major genes involved in oil biosynthesis.

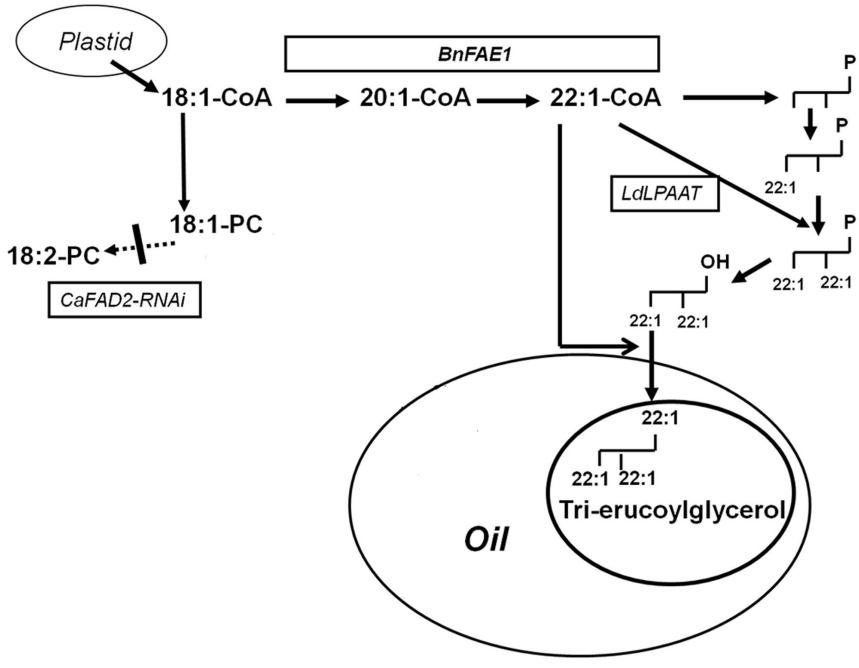

Figure 4. Simplified diagram of strategies used in Paper I to promote the 22:1 accumulation as tri-erucoylglycerol

4.2 Major methods

Agrobacterium was used in this thesis for genetic transformation of crambe using hypocotyls as explants and kanamycin selection to generate transgenic lines with the target genes (Papers I and III). In all transformation vectors, the target genes were under the seed-specific napin promoter (*fp1*). All transgenic lines were analyzed using molecular methods, such as PCR, Southern blot or qRT-PCR, to confirm transgenic events, verifying transgene copy number and analyze gene expression levels (Papers I, II and III). For cloning work, PCR with degenerate primers, rapid amplification of cDNA ends (RACE) and fusion

PCR were used to clone the candidate genes and to generate multiple-gene expression cassettes (Paper III). Gateway^{TM} recombinant technique was employed for constructing the RNAi expression vectors (Paper III). The oil composition was analyzed using gas chromatography (GC) in half-seed or whole seeds (without pod) (Paper I, II, III), and in seeds with pods (Paper IV). *In vitro* studies of enzyme activities were carried out using microsomal preparations from developing embryos. *In vivo* [^{14}C] glycerol labeling was conducted with the developing embryos (Paper IV) and different lipids were separated by thin layer chromatography (TLC) (Papers I and IV). For enzyme activity studies, chemical synthesis, isolation and quantification of ^{14}C labeled substrates were employed (Paper IV). A transcriptomic approach was used for profiling gene expression and for identifying key or novel genes involved in the oil biosynthesis and RNA sequencing was performed using Illumina sequencing technique (Paper V).

4.3 General results and discussion

In **Paper I**, three different transformation vectors were introduced into crambe. These three vectors were: i) *BnFAE1+LdLPAAT*; ii) *CaFAD2-RNAi*; and iii) *BnFAE1+LdLPAAT+CaFAD2-RNAi*. Stable transgenic lines were recovered from all three vectors. The lines with *BnFAE1-LdLPAAT* and *FAD2-RNAi* showed a moderate increase and no increase in 22:1 content, respectively. However, the most significant increase in 22:1 was found in transgenic lines with all three target genes with an average of 71% 22:1 in the seed oil in the T_3 generation.

In **Paper II**, a hygromycin-based selection system was successfully established for future crambe retransformation. Furthermore, the protocols for molecular evaluation of transgenic lines (Southern blot and qRT-PCR analysis) were optimized in this paper. Appropriate sampling time during seed development for gene expression analysis by qRT-PCR was studied concerning the *FAE1* and *FAD2* genes and it was found that the gene expression level was highest at 20 days after flowering. A suitable reference gene for crambe qRT-PCR analysis was also discussed in this paper.

As mentioned earlier, since the PC-related metabolism might contribute to oleic acid and PUFA accumulation in crambe seeds, disruption of the PC pathway-related enzymes LPCAT, PDCT and PDAT may increase the precursor 18:1-CoA, thus further increasing the 22:1 content in GM crambe (Figure 5). To verify the functions of these three enzymes in seed oil biosynthesis in crambe, we first cloned the candidate genes from WT crambe and prepared RNAi expression vectors with these genes either alone or in

combination. These vectors were then transformed into WT crambe to evaluate their functions related to observed FA compositions (Paper III).

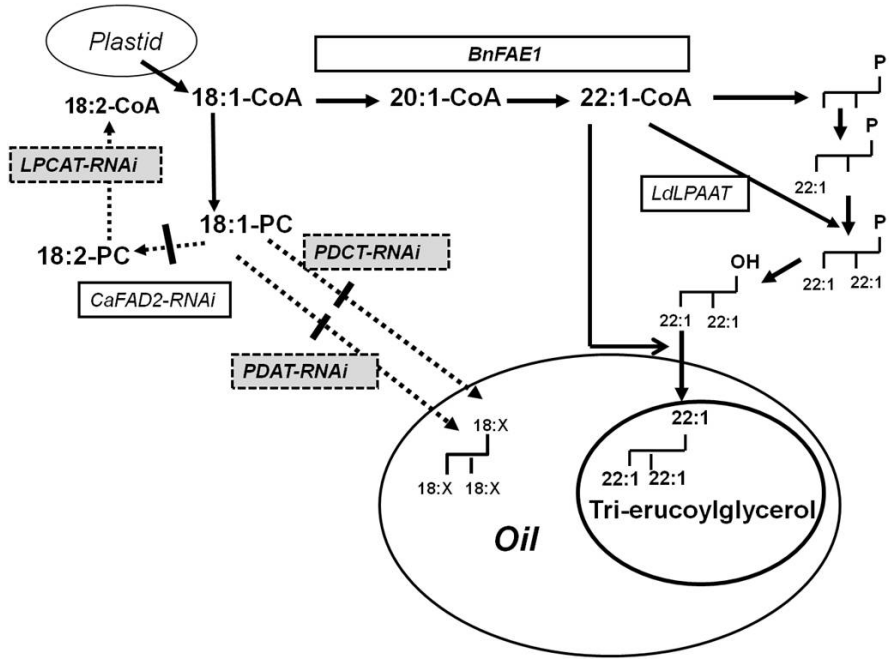

Figure 5. Hypothetical strategy for further increasing 22:1 accumulation in GM crambe.

The main results of **Paper III** include: i) The candidate genes of *LPCAT, PDCT* and *PDAT* genes with full length coding sequence were cloned from the developing seeds of WT crambe; ii) the most conservative region within different isoforms of the same candidate gene was used to construct the RNAi expression vectors for crambe transformation; iii) all RNAi transgenic lines showed a trend of significantly increased 18:1, especially in the *RNAi-LPCAT-PDCT* lines. However, the transgenic lines with increased 18:1 level showed no increase in 22:1. This is expected as the carbon chain elongation enzymes were not altered and the gene for effective incorporation of 22:1 at the *sn*-2 position was not integrated in either of these lines. Both *RNAi-LPCAT* and *RNAi-LPCAT-PDCT* even showed a negative impact on 22:1 accumulation, but no decrease in 22:1 was observed in *RNAi-PDCT* and *RNAi-PDAT* lines. Any increase in the 18:1 pool without a decrease in 22:1 levels would indicate new potential for a further increase in 22:1 contentl in the ultra-high 22:1 GM line. Based on the results from Papers I and III, it can be concluded that a combination of down-regulation of *CaPDCT* and *CaPDAT* together with

expression of the *LdLPAAT* would probably increase 22:1 levels more in crambe than these genes alone. This could be studied in the future.

Moreover, a *RNAi-LPCAT-PDCT-PDAT* expression vector with kanamycin selection was prepared and transformed into WT crambe, but the results were not included in Paper III for two reasons: 1) No stable line was obtained, due to time limitations; and 2) it was difficult to interpret the patterns of phenotype changes regarding FA composition, probably due to interaction of these three genes, leading to a possible compensation mechanism (Wang *et al.*, 2012)

In **Paper IV**, both *in vitro* and *in vivo* studies were conducted to evaluate the activities of some major enzymes involved in seed oil biosynthesis. Microsomal preparations from GM and WT crambe, as well as other plant species, were used in the *in vitro* study, in which different substrate combinations featuring the enzymatic activities involved in the Kennedy pathway were examined. The *in vivo* study was conducted with labeling of [^{14}C]glycerol in WT crambe in comparison to WT rapeseed. Lipid profiling featuring the 22:1 accumulation in TAG, DAG, PC and total oil accumulation during the different seed developmental stages was conducted in both GM and WT crambe. The results confirmed that introduction of *LdLPAAT* into crambe has indeed facilitated incorporation of 22:1-CoA at the *sn*-2 position of TAG, as evidenced by its high activity and a significant increase in 22:1 level. Low PC-DAG inter-conversion was observed in both WT and GM crambe, indicating relatively low PDCT activity in crambe compared with Arabidopsis. Lower oil accumulation and slightly delayed plant development of the GM line compared with WT were observed. Moreover, delayed accumulation of 22:1 in seed oil but continuously higher accumulation of 22:1 in DAG in GM crambe than WT was detected at 20 days after flowering (DAF) (Figure 6).

In **Paper V**, a comparative transcriptome study of high 22:1 GM and WT crambe was conducted with the seeds sampled following the time-course of seed development. In order to construct a reference library for this comparison, all types of crambe tissues were used. Thus we have constructed the first *de novo* assembly reference library for crambe. All three target trans-genes reported earlier in Paper I were expressed, as expected. Candidate genes with several gene isoforms isolated in Paper III and expected to be involved in PC metablism were analyzed regarding their expression profiles in both WT and GM crambe. Differential expression of these genes was evident in WT and GM crambe. Different isoforms of the candidate genes encoding DGAT1 and DGAT2 were found in the *de novo* assembly database. The expression of genes encoding putative DGAT1 and DGAT2 in WT and GM crambe is shown in Figure 7. It was assumed that DGAT2 could be the enzyme with high specificity for erucoyl-CoA incorporation into TAG. Some transcription factors

(TFs) related to the TAG biosynthesis and oil accumulation in seed were also found among crambe unigenes. Paper V generated a large quantity of genetic information in crambe and provides a rich data source for further studies of the molecular and metabolic mechanisms underlying the accumulation of 22:1 rich seed oils.

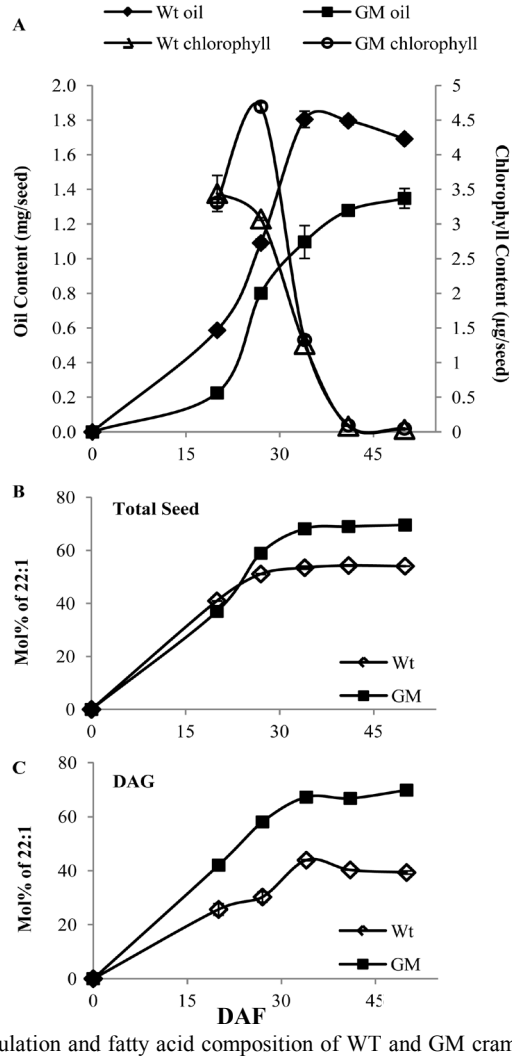

Figure 6. Oil accumulation and fatty acid composition of WT and GM crambe seeds at different days after flowering (DAF). (A) Oil content and chlorophyll content; (B) 22:1 content in total seed oil; (C) 22:1 content in diacylglycerols (DAG).This figure is reproduced with the permission (Guan *et al,*2014)

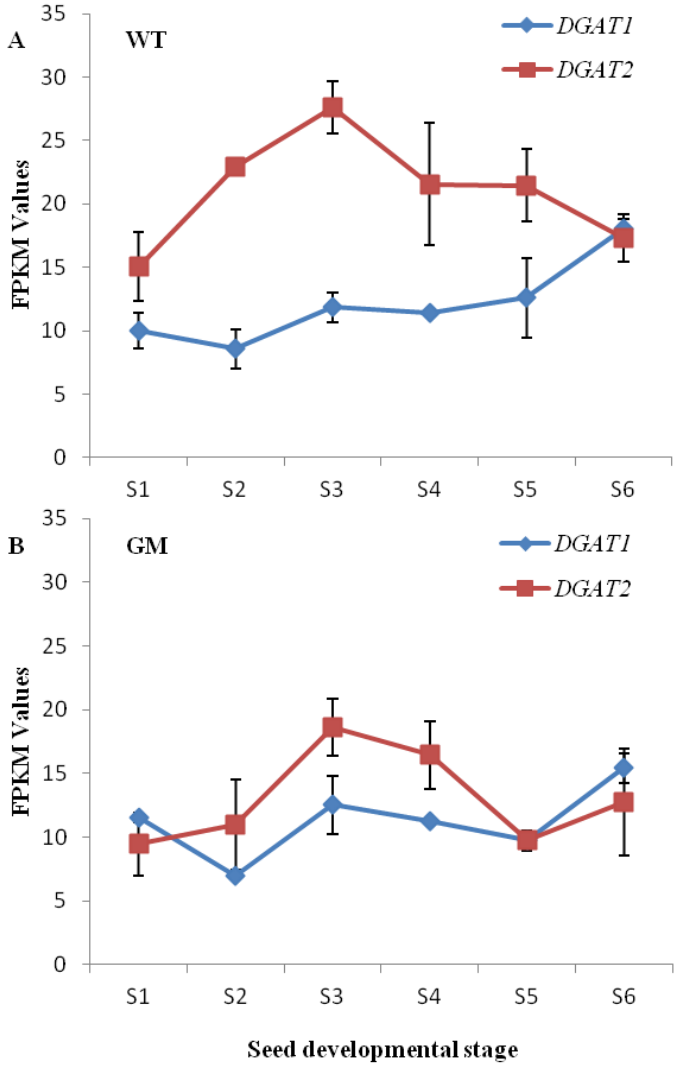

Figure 7. Transcript levels (fragments per kilobase of exon per million fragments mapped, FPKM) in (A) wild type (WT) crambe and (B) genetically modified (GM) crambe corresponding to DGAT1 (Acyl-CoA:diacylglycerol acyltransferase, *Arabidopsis thaliana* At2g19450) and DGAT2 (Acyl-CoA:diacylglycerol acyltransferase, *Arabidopsis thaliana* At3g51520) during the seed development defined by days after flowering (DAF). S1(1-5 DAF), S2(8-12 DAF), S3(15-19 DAF), S4 (20-24DAF), S5 (25-29 DAF), S6 (30-34 DAF).

5 Conclusions and future perspectives

Although we were successful to produce a stable GM crambe line with 73% of erucic acid, this line had some undesirable characteristics such as slightly slower seed development and lower oil content than WT (Paper I and IV). It is of prime importance to identify the causes of these problems. Here are some clues about how to solve these problems:

1) In GM crambe, slower oil accumulation than in WT crambe was observed at early stages. A higher amount of erucic acid in DAG in GM than WT and a similar amount in TAG at early stages (20 DAF) indicate deficiency in acylating DAG with 22:1-CoA. This could be due to the lack of a DGAT2 that is probably specific for erucoyl-CoA, as 22:1-CoA has been shown to be dominant (77%) in the acyl-CoA pool at an early stage (12 DAF) (Furmanek *et al.*, 2014). Since an increase in specific activity in DGAT activity for 22:1-CoA, but not for 16:0-CoA and 18:1-CoA, in microsomal preparations of crambe WT seeds in mid development has been reported (Furmanek *et al.*, 2014), it can be speculated that an erucoyl-specific DGAT is induced in expression at that developmental stage. Our transcriptome data showed that at this stage the expression of DGAT2 peaked (Paper V), suggesting that DGAT2 might be this erucoyl-specific DGAT. Thus expressing DGAT2 with a promoter that induces expression early in seed development in the GM crambe might alleviate the low oil content and low erucic acid content observed at the early stages of development in these seeds (Paper IV).

2) From mid-stage (27-50 DAF) of oil accumulation, GM crambe accumulates 86 mol % of 22:1 in the oil, compared with 68 mol % of 22:1 in the oil at earlier stages (20-27 DAF) (Paper IV). Accumulating 86 mol% of 22:1 in the mature seeds of GM crambe should be achievable if the bottleneck at the earlier stages can be identified. Oleoyl-PC has been suggested to contribute to the formation of VLCFA in other oil crops of the Brassicaceae

(Bao *et al.*, 1998). Thus some of the precursor (18:1) for elongation might have to pass through PC to be efficiently elongated in crambe. The decrease in 22:1 seen in the lines expressing RNAi-LPCAT seems to support such a speculation (Paper III)

3) Introduction of a WRI1 regulated promoter could facilitate to increase the expression of transgenes (Paper V).

4) With regard to the incorporation of 22:1 to the *sn*-1, *sn*-2 and *sn*-3 position, expression of GPAT, LPAAT and DGAT with higher substrate specificity for 22:1 substrates would be desirable.

Large-scale biological studies and computational biology approaches such as the '-omics' studies have generated huge amounts of genetic information for identifying novel genes and pathways. While these data by themselves cannot answer any questions, they provide valuable background information for targeted biochemical and metabolic studies (Szymanski *et al.*, 2014).

The emerging site-mutagenesis techniques, such as the Clustered Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) system (Jinek *et al.*, 2012), could be applied in the future metabolic engineering of crambe seed oils as an alternative to RNAi in certain cases where seed-specific genes need to be silenced and for functional analysis of complex gene family members.

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