University of Alberta

Role of Cytosolic Acyl-CoA Binding Protein in Seed Oil Biosynthesis

by

Olga Yurchenko

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Plant Science

Department of Agricultural, Food and Nutritional Science

Fall 2009
Edmonton, Alberta

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Role of cytosolic acyl-CoA binding protein in seed oil biosynthesis** in partial fulfillment of the requirements for the doctoral degree in **Plant Science**.

_____

Dr. Randall Weselake
(Supervisor)

_____

Dr. Jocelyn Ozga

_____

Dr. Walter Dixon

_____

Dr. Enrico Scarpella

_____

Dr. John Thompson
(External Examiner)

DEDICATION

to my ***baba Masha*** –

a woman of little education but great knowledge,

my first mentor in Plant Science

ABSTRACT

Acyl-CoA binding protein (ACBP) ubiquitously found in eukaryotic organisms fulfills important functions of solubilisation, protection and transport of acyl-CoA esters, a major intermediate of lipid metabolism. This thesis presents an investigation of the physiological role of the small cytosolic ACBP in seed oil biosynthesis. The second important objective of this study was to evaluate the use of ACBP as a molecular tool for modification of seed oil content and/or fatty acid (FA) composition. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* and *Brassica napus* was performed with a number of genetic constructs designed for seed-specific expression of the *B. napus* cDNA encoding a small cytosolic ACBP. Protein level and subcellular localization of BnACBP in *A. thaliana* transgenic seeds depended on the structure of the genetic constructs – mainly, the presence of additional in-frame sequences, encoding a protein fusion partners or signal peptides. Seed oil from *A. thaliana* T_2 and T_3 seeds had increased polyunsaturated fatty acid (PUFA) percentage (18:2$^{cis\Delta 9,12}$ and, in some lines, 18:3$^{cis\Delta 9,12,15}$) at the expense of very-long-chain monounsaturated (20:1$^{cis\Delta 11}$) and saturated (18:0) fatty acids. An increase in PUFA levels in seed oil was due to enhanced acyl channeling from the acyl-CoA pool to phosphatidylcholine, the substrate for extraplastidial FA desaturation. The activity of *A. thaliana* acyl-CoA: lysophosphatidylcholine acyltransferase (AthLPCAT), an enzyme involved in acyl exchange between acyl-CoA and PC, was significantly increased in the presence of the recombinant *B. napus* ACBP (rBnACBP) in the reaction mixture. rBnACBP also modulated enzymatic activities of glycerol-3-phosphate

acyltransferase and diacylglycerol acyltransferase *in vitro*. Finally, the effect of constitutive or seed-specific gene silencing of *ACBP* on seed oil formation was examined. *A. thaliana* transformation with RNAi constructs resulted in partial suppression of *ACBP* expression and changes in FA composition of seed oil which included an increase in the percentage of $18{:}1^{cis\Delta 9}$ and $18{:}2^{cis\Delta 9,12}$ and, decrease of $18{:}3^{cis\Delta 9,12,15}$. Overall, the results of this study demonstrate that the small cytosolic ACBP plays an important role in acyl exchange between acyl-CoA and PC metabolic pools. Overexpression of ACBP during seed development can be useful in genetic engineering strategies aimed at modifying the FA composition of seed oils.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Dr. Randall Weselake for giving me the opportunity, and guidance and support throughout the PhD program, which made it a rewarding and memorable experience. His ideas, vision, and dedication are truly inspiring. I also acknowledge my supervisory committee members Dr. Jocelyn Ozga and Dr. Walter Dixon for their continuous support in this project: their timely assistance and feedback were truly invaluable through the program. I would like to express my gratitude to Dr. John Thompson (University of Waterloo) for serving as an external examiner at my thesis defence, and the out of department examiner Dr. Enrico Scarpella (Department of Biological Sciences, University of Alberta).

I am very much in debt to the members of the global research community, who contributed to this project by sharing their insights and experience and providing materials that made a world of a difference in my experiments: Dr. Matthew Hills (John Innes Centre, UK), Dr. Mee-Len Chye (University of Hong-Kong, China), Dr. Raymond Pacovsky (College of Alameda, Alameda, CA), Dr. Sten Stymne (Swedish University of Agricultural Sciences, Alnarp, Sweden) and Dr. Antoni Banas (University of Gdansk, Gdansk, Poland).

I am very fortunate to have been able to use the lab facilities of our industry partner SemBioSys Inc. (Calgary, AB), and I am thankful to the company's members Dr. Cory Nykiforuk and Dr. Maurice Moloney for their supervision and support during and beyond the time of my stay.

I very much appreciate all the help I received from the members of Dr. Weselake's group: research associates Elzbieta Mietkiewska, Rodrigo Siloto, Martin Truksa, project manager Chris Kazala, lab technician Robin Hryniuk and graduate students Qin Liu, Xue Chen, Jean Malcolm-McDonald, Halina Kremer and Tim Warrington. Special thanks go to the lab manager Crystal Snyder for the excellent technical, academic and mental support at all stages of the project.

I would like to acknowledge the Alberta Crop Industry Development Fund Student Development Program for providing funds for this project. Also, I appreciate all the work of the Department of Agricultural, Food and Nutritional Sciences and Faculty of Graduate Studies and Research staff in assisting with administrative aspects of the program.

And finally, I would like to thank my parents for trusting my judgement enough to let me pursue my dreams; my "baby" sister Lena for teaching me patience; and last, but not least, my wonderful husband Aaron for being here for me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
ACAT	acyl-CoA: cholesterol acyltransferase
ACBP	acyl-CoA binding protein
ACCase	acetyl-CoA carboxylase
ACP	acyl carrier protein
ACS	acyl-CoA synthetase
acyl-CoA	acyl-coenzyme A
AMP	adenosine monophosphate
ANT	adenine nucleotide translocase
AtEBP	*Arabidopsis thaliana* ethylene-responsive element-binding protein
AtFP6	*Arabidopsis thaliana* farnesylated protein 6
ATP	adenosine triphosphate
B82	DNA fragment encoding 82 N-terminal residues from BBE from *Papaver somniferum* (opium poppy)
BBE	berberine bridge enzyme
BnACBP	*Brassica napus* acyl-CoA binding protein
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary DNA
CoA	coenzyme A
CPT	choline phosphotransferase
D9 (scFv)	single chain variable fragment antibody specific for *A. thaliana* 18 kDa oleosin
DAF	days after flowering
DAG	*sn*-1,2-diacylglycerol
DBI	diazepam-binding inhibitor
DGAT	diacylglycerol acyltransferase
DGDG	digalactosyldiacylglycerol
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide-5'-triphosphate
DTT	dithiothreitol

EDTA	ethylene diamine tetraacetate
EST	expression sequence tag
ER	endoplasmic reticulum
FA	fatty acid
FABP	fatty acid binding protein
FAD	fatty acid desaturase
FAE	fatty acid elongase
FAME	fatty acid methyl ester
FAS	fatty acid synthase
g	gram
g	gravitational force
G3P	glycerol-3-phosphate
GA	gibberellic acid
GABA	gamma-aminobutyric acid
GC	gas chromatography
GFP	green fluorescent protein
GPAT	glycerol-3-phosphate acyltransferase
GST	glutathione-S-transferase
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HNF-4α	hepatocyte nuclear factor 4α
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
kb	Kilobase
Kd	dissociation constant
kDa	Kilo Dalton
L	liter
LB	Luria-Bertani broth
LB	left border
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyltransferase
LPC	lysophosphatidylcholine
LPCAT	lysophosphatidylcholine acyltransferase

LPE	lysophophatidylethanolamine
LPG	lysophophatidylglycerol
LPLAT	lysophopholipid acyltransferase
LTP	lipid transfer protein
M	molar
MAG	monoacylglycerol
MD	microspore-derived
µE	microEinstein
µg	microgram
mg	milligram
MGDG	monogalactosyldiacylglycerol
µL	microliter
mL	millilitre
µm	micrometer
µM	micromolar
mM	millimolar
mRNA	messenger RNA
MUFA	monounsaturated fatty acid
NADH	nicotinamide adenine dinucleotide reduced
NADPH	nicotinamide adenine dinucleotide phosphate reduced
NPTII	neomycin phosphotransferase II
OB	oil body
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
pat	phosphinothricin n-acetyl transferase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDAT	phospholipid: diacylglycerol acyltransferase
PE	phophatidylethanolamine
PG	phosphatidylglycerol
phaP	phaseolin promoter

phaT	phaseoline terminator
PI	phosphatidylinositol
PKM	proline knot motif
PL	phospholipid
PLA_2	phospholipase A_2
PM	plasma membrane
PP2A	protein phosphate 2A subunit
PPAR	peroxisome proliferator-activated receptor
PPT	phosphinothricin
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
rACBP	recombinant acyl-CoA binding protein
RB	right border
RNA	ribonucleic acid
RNAi	RNA interference
RT	room temterature
SCP-2	sterol carrier protein-2
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFA	saturated fatty acid
SREBP-1	sterol regulatory element-binding protein 1
TAG	triacylglycerol
T-DNA	transferred DNA
TLC	Thin layer chromatography
TSP	total seed protein
ubiP	ubiquitin promoter
ubiT	ubiquitin terminator
WT	wild-type

1. Introduction

Lipids are the major form of carbon storage in the seeds of many plant species, constituting up to ~60% of the dry weight of such seeds (Ohlrogge and Browse, 1995). In addition to being a major source of dietary fat in human and animal nutrition, seed oils are also being extensively investigated as feedstock for nutraceutical and industrial applications (Cahoon *et al.*, 2007; Davies, 2007). The major component of seed oil is triacylglycerol (TAG), consisting of a glycerol molecule esterified with three fatty acid (FA) moieties, which affect both the physical and nutritional properties of the oil. Modification of the FA composition of seed oil has been a goal in a number of studies aimed at producing 'designer' oils with the most desirable characteristics for a particular application (Cahoon *et al.*, 2007; Graham *et al.*, 2007; Napier, 2007). High demand and interest in the production of novel FAs has led to substantial progress in the identification of biochemical activities responsible for synthesis of desirable FAs in different organisms (Sayanova *et al.*, 2003; Huang *et al.*, 2004; Meyer *et al.*, 2004).

Many of the enzymes involved in seed oil biosynthesis require acyl-CoA, an activated form of FA, as a substrate (Weselake, 2005). The availability of certain acyl-CoAs may be an important factor influencing seed oil FA composition. Acyl-CoA binding protein (ACBP) has been identified as a pool former and transporter of the 'currency' of lipid metabolism – acyl-CoAs (Rasmussen *et al.*, 1994; Knudsen J. *et al.*, 1999). In plants, ACBP has been proposed to be involved in mediating stress responses to abiotic factors and defence reactions against

pathogens (Walz *et al.*, 2004; Chen *et al.*, 2008). ACBP is expressed at high levels in developing seeds during seed oil accumulation, but its physiological role in seed oil biosynthesis remains unknown (Engeseth *et al.*, 1996). The ultimate goal of this project was to investigate the physiological role of ACBP in seed oil biosynthesis and evaluate the use of this protein as a molecular tool for modification of seed oil FA composition and/or content. The project was based on the following specific objectives:

1. To genetically modify *Arabidopsis thaliana* to produce small cytosolic ACBP from *Brassica napus* at different sub-cellular sites during seed development and evaluate the effect(s) of this modification on seed oil content and FA composition of the transgenic seeds.

2. To study effect(s) of the recombinant small cytosolic ACBP from *B. napus* on the activity of acyltransferases involved in seed oil biosynthesis using *in vitro* assays performed with microsomal fractions of *B. napus* cell suspension cultures or yeast cultures producing recombinant acyltransferase.

3. To down-regulate the expression of small cytosolic *ACBP* in *A. thaliana* through RNA interference (RNAi) technology and evaluate the effect(s) of *ACBP* suppression on seed oil content and FA composition.

The results of this study demonstrate that small cytosolic ACBP plays an important role in acyl exchange between acyl-CoA and phospholipid metabolically active pools, promoting FA channeling towards desaturation. Transgenic plants over-expressing *ACBP* in developing seeds accumulated a higher percentage of polyunsaturated FAs in the seed oil at the expense of very-

long chain monounsaturated FA. Small cytosolic ACBP may prove to be a useful molecular tool in genetic engineering strategies aimed at modifying the FA composition of seed oils.

2. Literature review

2.1. Acyl-CoA

2.1.1. Acyl-CoA structure and physical properties

Long-chain acyl-coenzyme A (acyl-CoA) is an amphipathic molecule that is a major intermediate of lipid synthesis and has a number of other functions in a living cell. It has a hydrophilic head group (CoA), and a hydrophobic tail (the acyl chain), as illustrated in Figure 2.1. The acyl chain can vary in length, degree of unsaturation and presence or absence of additional groups (hydroxyl-, epoxy-, etc.). Acyl groups common to most land plants are C16-20 long and have 0-3 double bonds.

Because of the amphipathic nature, long-chain esters can form micelles at high concentrations, which may decrease their availability to acyltransferases. The critical micellar concentration for acyl-CoAs ranges between 3-150 μM depending on the acyl-chain and salt concentration of the solution (Faergeman and Knudsen J., 1997). Acyl-CoA esters can also partition into phospholipid vesicles and microsomal membranes by insertion of the acyl chain into the bilayer, which can affect membrane integrity (Boylan and Hamilton, 1992; Banhegyi *et al.*, 1996).

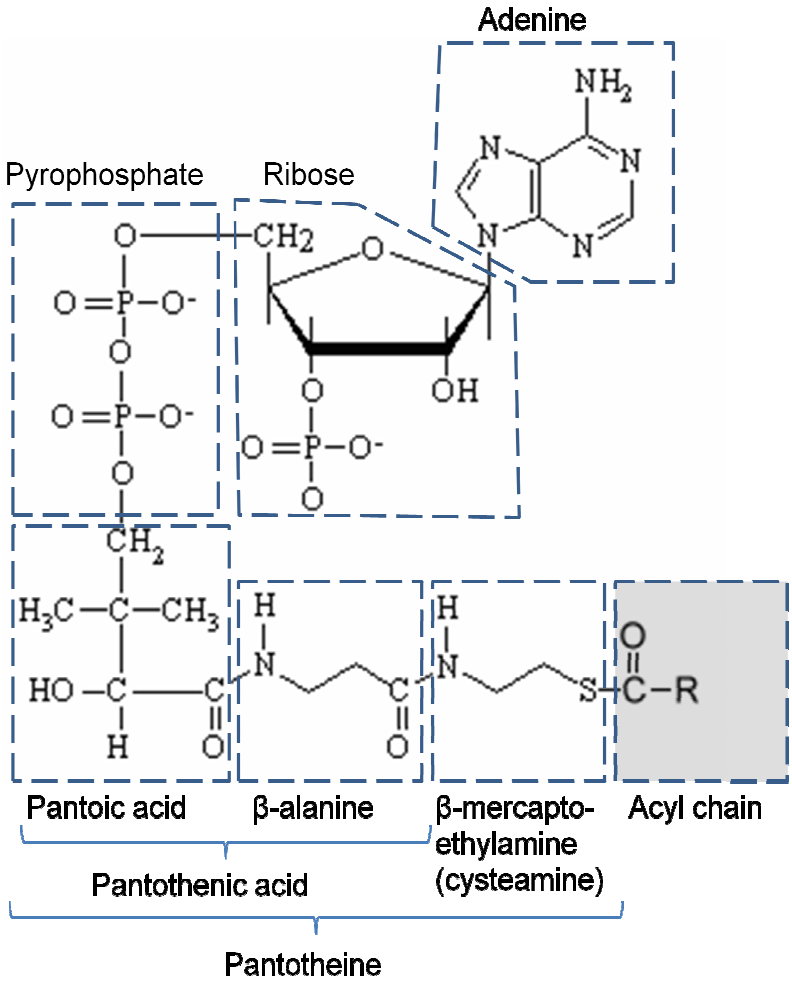


Figure 2.1. Chemical structure of the acyl-CoA ester. Acyl group (hydrophobic part of the molecule) is shaded in grey.

2.1.2. Synthesis of acyl-CoAs in plants

The acyl chains which become esterified to CoA are derived from plastidial fatty acid (FA) biosynthesis. FA biosynthesis starts with the formation of malonyl-CoA from acetyl-CoA and bicarbonate catalyzed by the enzyme acetyl-CoA carboxylase (ACCase, EC 6.4.1.2). Malonyl-CoA produced in this reaction is the central donor of the carbon blocks for FA synthesis. A major unresolved question in plant metabolism is how the pool of acetyl-CoA is generated. Glucose-6-phosphate and phosphoenolpyruvate have been proposed as the major precursors of the plastidial acetyl-CoA pool in oilseeds (Fox *et al.*, 2000b; Kubis *et al.*, 2004; Schwender *et al.*, 2006). Coenzyme A itself is synthesized from pantothenate in five enzymatic reactions (Falk and Guerra, 1993; Kupke *et al.*, 2003).

FA synthesis occurs through consecutive additions of two carbon units to the nascent acyl chain, catalyzed by FA synthase complex. Plant FA synthase is dissociable (type II) and consists of at least 8 separate proteins (Shimakata and Stumpf, 1982; Slabas *et al.*, 2001). In the first cycle of reactions catalyzed by KASIII, butyryl-ACP is produced, and the addition of two carbon units from malonyl-ACP continues until the acyl chain length reaches C16 or C18. The condensation reactions from C4 to C16, and C16 to C18 are catalyzed by KAS I and KAS II, respectively. The acyl chain is covalently attached to acyl carrier protein (ACP) during this process. A soluble plastidial stearoyl-ACP desaturase (EC 1.14.19.2) can introduce a double bond at the Δ9 position, resulting in oleic acid ($18{:}1^{cis\Delta 9}$) (Jaworski, 1987). The desaturase reaction requires, in addition to a

stearoyl-ACP, a source of electrons and molecular oxygen. Reduced ferredoxin has been implicated as the immediate donor of electrons to the desaturase. In nonphotosynthetic tissue, NADPH is the most likely source of electrons.

The biosynthesis of FA in the plastids is terminated when the acyl group is removed from ACP by the catalytic action of acyl-ACP hydrolase and released into the cytosol (thioesterase, EC 3.1.2.14). Alternatively, acyl-ACP can be utilized by acyltransferases in the plastid for synthesis of the membrane lipids. The export of the acyl group through the plastid membrane into the cytosol is directly coupled to acyl-CoA synthesis. On the outer membrane of the plastid envelope, an acyl-CoA synthetase (ACS; EC 6.2.1.3) catatized the assembly of acyl-CoA thioesters, which become available for further modifications in the cytosol and for utilization in acyltransferase reactions to form glycerolipids in the ER. On the outside of the plastid, acyl-CoAs interact with cytosolic acyl-CoA binding proteins (ACBP), which facilitate maintenance, protection and transport of the acyl-CoA pool in the cell. It has been proposed that ACBP is involved in acyl-CoA export from the outer envelope of the plastids (Ohlrogge and Browse, 1995; Johnson *et al.*, 2002).

2.1.3. Utilization of acyl-CoAs in developing seeds

Once in the cytosol, newly synthesized acyl-CoA may be utilized in a variety of metabolic reactions: they can be further elongated, or esterified to the glycerol backbone to form glycerolipids where they may undergo further desaturation, or become degraded and recycled to meet the metabolic needs of the cell (Figure 2.2).

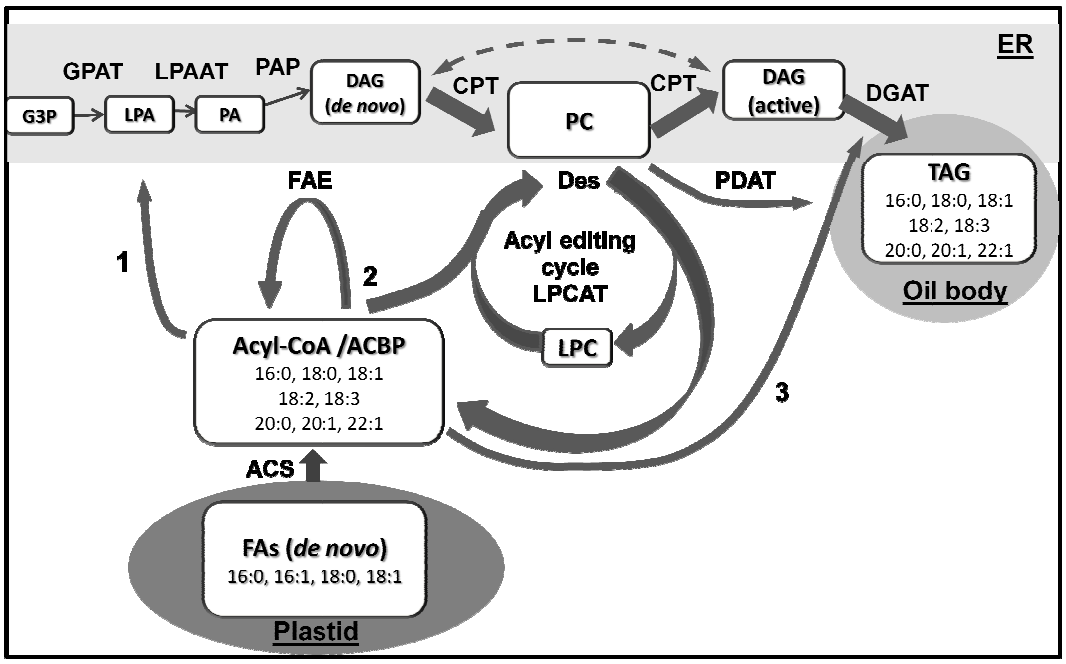


Figure 2.2. Utilization of acyl-CoA in the cytosol in developing seeds (Based on Bates *et al*., 2009). 1 – acyl channelling for *de novo* DAG synthesis; 2 – acyl channelling for remodelling: elongation and desaturation; 3 – acyl channelling for DAG acylation; ACBP – acyl-CoA binding protein; ACS – acyl-CoA synthetase; CPT – cholinephosphotransferase; DAG – diacylglycerol; Des – desaturases; DGAT – diacylglycerol acyltransferase; FA – fatty acid; FAE – fatty acid elongase; G3P – glycerol-3-phosphate; GPAT – glycerol-3-phosphate acyltransferase; LPA – lisophosphatidic acid; LPAAT – lysophosphatidic acid acyltransferase; LPC – lysophosphatidylcholine; LPCAT – lysophosphatidylcholine acyltransferase; PA – phosphatidic acid; PAP – phosphatidic acid phosphatase; PC – phophatidylcholine; PDAT – phospholipid: diacylglycerol acyltransferase; TAG – triacylglycerol.

In developing soybean embryos, more than a half of the acyl chains that enter the cytosol as *de novo* acyl-CoAs, are incorporated into the phospholipids (mainly phosphatidylcholine, PC) of the ER membrane (Bates *et al.*, 2009). This incorporation is catalyzed by acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT; EC 2.3.1.23), which is considered to be one of the most active enzymes in seed oil biosynthetic pathways (Stymne and Glad, 1981; Ichihara *et al.*, 1995). Acyl groups esterified to the *sn*-1 and *sn*-2 position of PC become available as substrates to the membrane-bound desaturases (Jackson *et al.*, 1998). Δ12-desaturase (EC 1.14.19.6) and Δ15-desaturase (EC 1.14.19.3) catalyze the introduction of the second and the third double bonds to 18:1, producing linoleic ($18:2^{cis\Delta 9,12}$) and α-linolenic acids ($18:3^{cis\Delta,9,12,15}$) (Miquel and Browse, 1992; Browse *et al.*, 1993).

Desaturated FA can be returned back to the acyl-CoA pool, or can be channelled to other glycerolipids *via* acyl-CoA independent mechanisms. There is no well defined mechanism for how polyunsaturated acyl groups get transferred from PC to acyl-CoA. One possible way known as Lands cycle involves deacylation of PC at *sn*-2 position with phospholipase A2 and FA activation with acyl-CoA synthetase, while LPC gets reacylated by LPCAT (Lands 1960; Das *et al.*, 2001). This process requires ATP and CoA for acyl-CoA formation. ATP-independent acyl-CoA synthesis was also observed and is believed to be driven by the reverse reaction of LPCAT, which produces acyl-CoA and lysophosphatidylcholine (LPC) (Stymne and Stobart 1984). Because the forward LPCAT reaction is more thermodynamically favourable, LPC gets quickly

acylated using another acyl-CoA. The combination of the forward and reverse reactions of LPCAT might be responsible for the continuous acyl exchange between PC and acyl-CoA which increases the levels of unsaturated FAs in the acyl-CoA pool (Stymne and Glad, 1981; Stymne and Stobart, 1984). *In vitro* studies have shown that a small protein capable of acyl-CoA binding (like BSA) was essential for the acyl exchange, while the reaction did not require the ATP-dependent acyl-CoA synthetase (Stymne and Glad, 1981; Stymne and Stobart, 1984).

A second possible mechanism is the so-called Land's cycle, which involves deacylation of PC at *sn*-2 position by phospholipase A_2 (PLA_2) and FA activation with the cytosolic ACS (Lands, 1960; Das *et al.*, 2001). This process requires ATP and CoA for acyl-CoA formation. The LPC resulting from PLA_2 deacylation gets re-acylated back to PC by LPCAT to complete the cycle.

Another modification of acyl-CoA on the membranes of the ER involves an elongation of the acyl chain with another one or more C2-blocks that come from the cytosolic malonyl-CoA (Leonard *et al.*, 2004). Elongation occurs through four enzymatic activities catalyzed by fatty acid elongase (FAE), in a fashion similar to FAS (Domergue *et al.*, 2000; Blacklock and Jaworski, 2006). In oleaginous plants elongation of FAs usually does not go further than C22.

Both plastidial-derived and modified FA moieties make up the cytosolic acyl-CoA pool, which is a source of substrates for the membrane-bound acyltransferases of triacylglycerol (TAG) biosynthesis, the major storage lipid in developing seeds (Weselake, 2002, 2005). A TAG molecule consists of a

'glycerol backbone' esterified with three acyl groups (Weselake, 2000). A major route of TAG biosynthesis is believed to be a series of consecutive acyl-CoA dependent acylations of the glycerol backbone, derived from *sn*-glycerol-3-phosphate (G3P). This pathway is known as a *sn*-glycerol-3-phosphate (G3P) or Kennedy pathway. The first acylation of G3P at the *sn*-1 position catalyzed by *sn*-glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15) produces lysophosphatidic acid (LPA), which gets acylated at *sn*-2 position by the catalytic action of lysophosphatidic acid acyltransferase (LPAAT, EC 2.3.1.51) to give phosphatidic acid. Before the final acylation of the glycerol backbone, the phosphate group is removed from PA by the catalytic action of PA phosphatase (PAP, EC 3.1.3.4) to form *sn*-1,2-diacylglycerol (DAG). The final reaction of the Kennedy pathway catalyzed by acyl-CoA: diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) results in the production of TAG.

sn-1,2-DAG is also a substrate for choline phosphotransferase (CPT, EC 2.7.8.2) which catalyzes the formation of phosphatidylcholine (PC). Recently some data from acyl flux studies in developing soybean embryos brings into question the linearity of the Kennedy pathway (Bates *et al.*, 2009). It is proposed that *de novo* DAG, synthesized in the first three reactions of the G3P pathway, is mostly used for PC synthesis, while DAG used in the final reaction of TAG synthesis is derived mainly from PC. DAG can be produced from PC through the action of phospholipases C. Also, reversibility of CPT has been proposed, and may contribute to the formation of DAG (Stobart and Stymne, 1985). Existence of kinetically distinct DAG pools has also been proposed based on the *in vitro*

studies (Vogel and Browse, 1996). Whether these different pools of DAG are connected spatially or metabolically still remains unknown.

Acyltransferases of the Kennedy pathway display unique substrate specificities, which are reflected in the positional distribution of acyl moieties on the glycerol backbone (Reviewed in Snyder *et al.*, 2009). GPAT has a relatively broad specificity, utilizing both saturated and unsaturated acyl donors (Christie *et al.*, 1991; Lísa and Holčapek, 2008). Microsomal LPAAT has the highest substrate stringency of the three Kennedy pathway acyltransferases (Laurent and Huang, 1992). Most plant LPAAT exclude saturated and very long chain acyl from the *sn*-2 position (Bernerth and Frentzen, 1990; Brown *et al.*, 2002). DGAT can incorporate a broad range of molecular species of acyl-CoA, but the substrate specificities can vary depending on the plant species (Taylor *et al.*, 1992; Vogel and Browse, 1996; Lung and Weselake, 2006).

The final acylation of the glycerol backbone at the *sn*-3 position can be also accomplished in acyl-CoA-independent reactions (Stobart K. *et al.*, 1997; Dahlqvist *et al.*, 2000). Acyl groups can be transferred to DAG directly from a phospholipid (PC or PE) or from another DAG molecule in the reaction catalyzed by phospholipid: diacylglycerol acyltransferase (PDAT) (Dahlqvist *et al.*, 2000; Banas *et al.*, 2000; Ghosal *et al.*, 2007).

As TAG continues to be synthesized by the membrane-bound acyltransferases, it accumulates between the phospholipid layers of the ER membrane (Wanner and Theimer, 1978; Huang, 1994). When the amount of TAG reaches a certain critical value, it pinches off as an oil droplet enclosed in a half-

unit membrane composed of phospholipids and proteins (Huang, 1996). This droplet ranging in size from 0.2 to 2.5 μm becomes an independent organelle called an oil body (OB) or oleosome. An OB maintains a high surface/volume ratio, thereby making seed lipid stores easily accessible for re-mobilization during seed germination (Huang, 1996).

Oleosin is the major protein found on the surface of OB. Synthesized by ER-bound ribosomes, oleosin is co-translationally inserted into ER membrane (Beaudoin *et al.*, 1999; Beaudoin and Napier, 2000) and gets rapidly transferred to the forming OB (Hills *et al.*, 1993). The main OB targeting signal on the oleosin is the central hydrophobic domain, which acquires a stable conformation in the hydrophobic environment of the oil body's core (Abell *et al.*, 2002). Oleosins form a protective barrier on the OB surface against the cytosolic phospholipases and prevent the OB coalescence during seed desiccation. Oleosins might also be important as docking sites for triacylglycerol lipase during seed germination (Huang, 1996).

2.1.4. Degradation of acyl groups during seed germination

TAG accumulated in oil bodies during seed maturation is used to fuel seed germination and seedling growth (Reviewed in Graham, 2008). First, TAG is hydrolyzed by an oil body-associated lipase to produce free FAs and glycerol. The FAs then enter glyoxysomes, where they get catabolised in β-oxidation. Whether FAs released from TAG lipolysis are activated by cytosolic ACS and transported as acyl-CoAs or remain free FAs is debatable. It is clear, however, that for β-oxidation in glyoxysomes FAs are to be converted to acyl-CoAs. β-oxidation,

similarly to FA biosynthesis, involves a series of enzymatic steps that proceed collectively in a repetitive fashion. Each round of β-oxidation yields a two-carbon block, acetyl-CoA, which is further metabolized in the glyoxylate cycle, and contributes to hydrocarbon production in the cytosol or respiration in mitochondria.

2.1.5. Functions of Acyl-CoA

Acyl-CoA esters are central intermediates in the synthesis and breakdown of FAs and complex lipids. Besides being precursors for biosynthesis of glycerolipids, acyl-CoAs are also involved in plant cuticular wax formation in epidermal cells (Reviewed in Kunst and Samuels, 2003). Acyl-CoA esters used for wax biosynthesis are characterized by a very-long acyl chain (C24-34), much longer than most acyl groups found within the cell.

Acyl-CoAs can allostericaly regulate a number of enzymes involved in acyl-CoA metabolism. First, excess acyl-CoA in the cytosol triggers the suppression of FA synthesis. Long-chain acyl-CoA esters inhibit acetyl-CoA carboxylase directly in yeast and in rat liver (Ogiwara *et al.*, 1978; Faergeman and Knudsen J., 1997). 18:1-CoA, even at relatively low concentrations (~1μM), inhibits the glucose-6-phosphate transporter in *B. napus* plastids, which reduces the carbon supply for FA biosynthesis (Fox *et al.*, 2000b). In mammals, ACS activity is tightly controled by the negative feedback regulation (Rasmussen *et al.*, 1993).

A relatively novel function described for acyl-CoAs is an activity in transcriptional regulation. Supplementation of 18:1 in the medium to *S. cerevisiae*

caused suppression of the transcription of the *OLE1* gene encoding Δ^9-desaturase activity (Choi *et al.*, 1996). Long-chain acyl-CoA esters were also demonstrated to interfere with the binding of FadR transcription factor to DNA containing the *fadB* promoter (DiRusso *et al.*, 1992). In mammalian cells, acyl-CoAs enter the nucleus and act as ligands for the nuclear receptors [peroxisome proliferator-activated receptor-α (PPAR α) and hepatocyte nuclear factor 4α (HNF4α)], modulating their transcriptional activity (Hertz R. *et al.*, 1998; Schroeder *et al.*, 2008). Cytoplasmic lipid binding proteins (FABP, ACBP) enhance acyl-CoA transport into nuclei and channelling to the respective nuclear receptors.

Acyl-CoA plays an essential role in membrane budding and fusion processes (Pfanner N. *et al.*, 1990). Acyl-CoAs were shown to act as cofactors to a Golgi-associated protein factor required for transport between cisternae of the Golgi stack (Glick and Rothman, 1987). Protein acylation, which is a covalent modification with FA, has been shown to be an important determinant in proper protein sorting and subcellular localization (McCabe and Berthiaume, 1999). Protein modification with acyl groups is also involved in several aspects of cellular signalling (Wedegaertner *et al.*, 1995; Dunphy and Linder, 1998). Long-chain acyl-CoA esters have been shown to regulate cellular ATP-dependent ion channels (Larsson *et al.*, 1996; Rapedius *et al.*, 2005; Tucker and Baukrowitz, 2008).

2.1.6. Subcellular localization and concentrations of acyl-CoAs

Because the sites of synthesis and utilization of acyl-CoAs are spatially separated in the cell, acyl-CoAs are required to move through the cytoplasm either

freely or with assistance of some proteins. The latter mechanism is facilitated by cytosolic ACBP and offers a means of specific intracellular targeting for acyl-CoA. Immunolocalization studies of acyl-CoAs in Arabidopsis root cells showed that acyl-CoAs are mainly localized within or in close proximity to the ER membranes (Diakou *et al.*, 2002). Acyl-CoAs can be also found in plasma membrane, in mitochondria and the nucleus (Elholm *et al.*, 2000; Diakou *et al.*, 2002).

The total cellular concentration of acyl-CoA esters can vary significantly depending on the tissue and its metabolic status, and can range from 5-160 μM in mammalian tissue (Reviewed in Faergeman and Knudsen J., 1997). In *B. napus* developing embryos and in *Arabidopsis* seedlings acyl-CoA content was estimated to be in the range of 3 to 6 μM (Larson and Graham, 2001). The actual concentration of free cytosolic acyl-CoA esters, however, remains unknown, because of the unresolved compartmentalization issue.

Unless substantial quantities of acyl-CoA are specifically bound by cytosolic binding proteins, these levels of acyl-CoA would be toxic due to nonspecific binding to the membranes and subsequent inhibition of enzymes vital to cell function. The intracellular concentration of free unbound acyl-CoA esters, however, is tightly controlled and rarely reaches the critical levels. The feedback inhibition of acyl-CoA synthetase prevents over-production of acyl-CoAs to match the needs of the cell (Faergeman and Knudsen J., 1997). Another control factor for assuring low concentrations of long-chain acyl-CoA under *in vivo* conditions is the high activity of acyl-CoA hydrolases found in most subcellular

compartments. Intracellular membranes can also accommodate small amounts of acyl-CoA (~15 mol%) without disruption of the bilayer structure. But most importantly, ACBP has been identified as a major pool former of the cytosolic acyl-CoA, which alleviates the detrimental effects of free acyl-CoAs, and at the same time maintains the physiologically active acyl-CoA pool available for lipid metabolism.

2.2. Acyl-CoA binding proteins

2.2.1. History of the discovery/ Cloning of plant ACBP

ACBP was characterized for the first time as an endogenous polypeptide isolated from a rat brain with agonistic action on benzodiazepine receptors, and named diazepam-binding inhibitor (DBI) for its ability to competitively inhibit benzodiazepine binding to its recognition sites (Guidotti *et al.*, 1983). It was identical in amino acid (aa) and cDNA sequence to ACBP purified from bovine liver a few years later (Mogensen *et al.*, 1987).

The first plant *ACBP* cDNA clones were isolated from *B. napus* and *A. thaliana* based on the sequence similarity to animal ACBPs (Hills *et al.*, 1994; Pacovsky, 1996; Engeseth *et al.*, 1996). These small soluble proteins, consisting of 92 amino acid residues, shared high sequence similarity with the bovine ACBP. A cDNA encoding *B. napus* ACBP homologue was cloned from a cDNA library constructed from RNA isolated from developing seeds of oilseed rape.

Proteomics studies of the phloem sap from castor bean led to identification of the castor bean ACBP (Sakuth *et al.*, 1993), and cDNA was isolated based on a peptide sequence (Erber *et al.*, 1997). Two ACBP isoforms were isolated from

proembryogenic masses of *Digitalis lanata* Ehrh. by column chromatography and preparative HPLC (Metzner *et al.*, 2000).

As a result of the new genome sequencing projects and the availability of EST databases, ACBP sequences were identified in many agricultural crops. A cDNA encoding ACBP was identified in cotton in a mass sequencing project (Reddy *et al.*, 1996). The deduced amino acid sequence of cotton ACBP displayed 80% and 53% identity with ACBP from rapeseed and yeast respectively. Rice *ACBP* cDNA clone (R1908) was isolated and identified in the Rice Genome Research Program and displayed a high similarity (71-76% identity) of the deduced amino acid sequence to other plant ACBP (Zhang *et al.*, 1999).

Analysis of the full genome of *A. thaliana* led to the discovery of six members of the ACBP gene family. Five out of six *Arabidopsis* ACBP genes encoded proteins larger than those described previously, and contained additional domains. A transmembrane domain was identified in two *Arabidopsis* ACBPs (ACBP1 and ACBP2) (Chye, 1998). cDNA encoding a membrane-bound ACBP homologue has been also cloned from a rice and *Agave americana* L. (Kikuchi *et al.*, 2003; Guerrero *et al.*, 2006). Homologues of the membrane-associated ACBP have been proposed to exist in other plants, based on the cross-reactivity of the antibodies raised against *A. thaliana* ACBP1 with protein samples from developing seeds of *Brassica juncea, Solanum melongena*, lettuce seeds, and roots of carrot and potato (Chye *et al.*, 1999).

2.2.2. Ubiquity of ACBP in eukaryotes

ACBP homologs were identified in all four eukaryotic kingdoms (Animalia, Plantae, Fungi and Protista) and eleven eubacterial species (Burton *et al.*, 2005). The appearance of ACBP very early on in evolution points towards a fundamental role of ACBP in acyl-CoA metabolism.

Mammalian small cytosolic ACBP can be divided into three subfamilies with names based on the tissues wherein the encoding genes are most highly expressed: l-ACBP (liver), t-ACBP (testes), and b-ACBP (brain) (Kragelund *et al.*, 1999). A much larger protein of 533 aa with an ACBP domain was identified from the bovine brain. This protein contains a predicted C-terminal transmembrane domain, and is classified as a separate protein group (m-ACBP).

ACBP genes have been also identified in insects. ACBP gene was cloned from *Drosophila melanogaster* and shows 51 to 56% identity with the previously known ACBP proteins (Kolmer *et al.*, 1994). In the silkmoth, *Bombyx mori*, a distinct ACBP designated as *pgACBP*, was specifically expressed in the pheromone gland during pheromonogenesis (Matsumoto *et al.*, 2001). *ACBP* cDNA encoding 85 aa was also isolated from the cotton bollworm *Helicoverpa armigera* (Wang *et al.*, 2008). Similar to *ACBP* expression pattern in *B. mori*, *HaACBP* is expressed predominantly in the midgut. *HaACBP* is expressed during the larval period and is probably responsible for nutrient absorption.

Eight predicted genes with the potential to encode proteins containing an ACBP domain were indentified in the *C. elegans* genome (Larsen *et al.*, 2006).

One member encoded a protein with a C-terminal predicted transmembrane domain.

An *ACBP* homolog was cloned from the budding yeast *Saccharomyces cerevisiae* by Rose *et al.* (1992). The yeast gene contains no introns and encodes a polypeptide of 87 aa (including the initial methionine), identical in length to the human gene product with 48% conservation of aa residues. The completed fungal genomes of *Neurospora crassa, Magnaphorthe grisea, Gibberella zeae, Ustilago maydis* and *Saccharomyces pombe* revealed only a single ACBP sequence homologous to the *S. cereivisiae* ACBP.

An *ACBP* homologue from *Trypanosoma brucei* was cloned by Milne and Ferguson (2000). ACBP is an essential protein in the bloodstream form of *T. brucei* (Milne *et al.*, 2001). The African malaria mosquito parasite *Plasmodium falciparum ACBP* (*PfACBP*) sequence was identified through a sequence search in the *P. falciparum* genome database (van Aalten *et al.*, 2001). An *ACBP* gene was identified in the opportunistic protist *Cryptosporidium parvum* (Zeng *et al.*, 2006). The *CpACBP1* gene encodes a protein of 268 aa that is three times larger than typical ACBPs (~ 90 aa) of animals. The CpACBP1 protein consists of an N-terminal ACBP domain (90aa) and a C-terminal ankyrin repeat sequence (170 aa).

The eubacterial ACBP homologs were similar in size to the mammalian ACBP homologs, with size ranging from 84 to 89 aa. All of the ACBP-containing bacteria, with the exception of *Ralstonia eutropha* and *Palstonia metallidurans*, are known to be pathogenic to plants or animals, suggesting that an ACBP gene could have been acquired from a eukaryotic host by horizontal gene transfer.

2.2.3. ACBP gene structure

The structure of ACBP genes can be characterized based on the example of the *A. thaliana ACBP* gene family (Table 2.1). Small cytosolic 10 kDa ACBP (in some literature referred to as ACBP6) is encoded by a small gene (ca. 1 kb), which consists of 4 exons and 3 introns. Genes encoding membrane bound ACBP1 and ACBP2 consist of 6 exons and 5 introns, respectively (Chye *et al.*, 1999; Chye *et al.*, 2000). The *ACBP3* gene has 4 exons and 3 introns, just like the gene encoding cytosolic ACBP, but is larger (ca. 2 kb) (Leung *et al.*, 2004). *ACBP4* and *ACBP5* are the largest (ca. 4 kb), consisting of 18 exons and 17 introns (Leung *et al.*, 2004). *ACBP4* and *ACBP5* genes are highly homologous (81.4% identity). Interestingly, the DNA sequences encoding the acyl-CoA binding domain in each of the six genes lies within the first three exons.

The *ACBP* promoter displays structural components characteristic for housekeeping genes. Beta-oxidation boxes and inositol-choline regulatory elements, sequences implicated in the regulation of FA synthesis and beta-oxidation that have been located in the yeast *ACBP* promoter (Rose et al, 1992), but were not detected in the *Arabidopsis ACBP1* promoter. Analysis of the promoter region of the rat *ACBP* gene revealed the characteristics of typical housekeeping genes (absence of TATA box; multiple transcription start points; and a CpG island between nucleotide -500 and +200) (Mandrup *et al.*, 1993a). *ACBP* expression was detectable in all organs tested. Most notably, the expression was high in cells involved in secretion and various transport processes.

Table 2.1. Characteristics of *A. thaliana* ACBP family genes

ACBP	gene	GenBank accession no.	Gene structure	CDS (bp)	Spatial expression	Factors that induce gene expression	Reference
ACBP (ACBP6)	At1g31812	NM_102916	4 exons, 3 introns	279	All tissues (higher in dev. seeds and siliques)	Cold treatment, light (↓)	1, 5, 11
ACBP1	At5g53470	NM_124726	6 exons, 5 introns	1017	All tissues (higher in dev. seeds and siliques)	Pb(II) nitrate treatment	2, 3, 10
ACBP2	At4g27780	NM_118916	6 exons, 5 introns	1065	All tissues (higher in roots, stem and flowers)	Pb(II) nitrate treatment	4, 6, 7, 10
ACBP3	At4g24230	NM_118556	4 exons, 3 introns	1086	All tissues (higher in siliques and young shoots)		8
ACBP4	At3g05420	NM_202498	18 exons, 17 introns	2010	All tissues (higher in roots and seedlings)	ethylene precursor 1-aminocyclopropane-1-carboxylic acid, methyl jasmonate, *Botrytis cinerea,* light	8, 9, 11
ACBP5	At5g27630	NM_122645	18 exons, 17 introns	1947	All tissues (higher in siliques, young shoots and mature leaves)	light	8, 11

References:

1 - Chen *et al.* (2008)
2 - Chye (1998)
3 - Chye *et al.* (1999)
4 - Chye *et al.* (2000)
5 - Engeseth *et al.* (1996)
6 - Gao *et al.* (2008)
7 - Kojima *et al.* (2007)
8 - Leung *et al.* (2004)
9 - Li *et al.* (2008)
10 - Xiao *et al.* (2008a)
11- Xiao and Chye (2009)

2.2.4. ACBP expression pattern

A. thaliana ACBP genes are expressed in numerous tissues (roots, stem, leaves, flowers, siliques), supporting the notion of the housekeeping nature of these genes (Table 2.1, column six). In addition to being expressed throughout the plant, each *ACBP* gene member is also preferentially expressed in a certain plant organ. The gene encoding small cytosolic ACBP is expressed constitutively, but is the most abundant in developing seeds and siliques in *A. thaliana* and *B. napus* (Engeseth *et al.*, 1996; Pacovsky, 1996). In these tissues, the ACBP concentration was estimated to be within the range of 0.5 – 1.6 ng/mg protein. The total cellular concentration of the small cytosolic ACBP in *A. thaliana* and *B. napus* seeds was calculated to be around 46.4μM and 65.7μM, respectively (Pacovsky, 1996). But since the cytoplasm occupied only a part of the cell, the actual ACBP concentrations within the cell are expected to be higher. The amount of *B. napus* ACBP increased from torpedo stage onwards and was highest during the mid to late cotyledonary stages (250 μg/ g FW) when TAG is accumulated at the highest rates (Engeseth *et al.*, 1996; Brown *et al.*, 1998). At later stages of seed development during the desiccation phase, the amount of ACBP per embryo fell to almost undetectable levels. The amounts of acyl-CoA and ACBP in *B. napus* embryos from different developmental stages were found to be very similar (Fox *et al.*, 2000a).

The genes encoding membrane-bound ACBP1 and ACBP2 are also expressed in all plant organs with the expression levels of *ACBP1* higher in siliques and flowers, and *ACBP2* transcript accumulating at higher levels in roots

and flowers compared to other plant parts (Chye, 1998; Chye *et al.*, 2000; Kojima *et al.*, 2007). Western blot analysis revealed that ACBP1 was enriched in *Arabidopsis* seeds and not in the remaining tissue of the silique (Chye *et al.*, 1999). Overall, *ACBP2* transcript was at least 4-fold more abundant than *ACBP1*.

The expression of *ACBP3* was higher in siliques and young shoots than in mature plants (Xiao and Chye, 2009). The large cytosolic ACBP4 accumulated in roots and in leaves and stems of *Arabidopsis* young seedlings, with lower expression in the flowers and siliques (Li *et al.*, 2008). *ACBP5* expression, on the other hand, was slightly elevated in siliques, young shoots and mature leaves (Xiao and Chye, 2009).

Expression of some *ACBP* genes in *A. thaliana* can be regulated by the environmental factors (Table 2.1, column seven). Expression of 10 kDa ACBP is induced by cold treatment, resulting in elevated levels of both *ACBP* mRNA and protein in leaves and stalks (Chen *et al.*, 2008). Pb (II) nitrate treatment has been shown to increase the expression of *ACBP1* and *ACBP2* in roots (Xiao *et al.*, 2008a). Another gene highly expressed in roots, ACBP4, was induced by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, methyl jasmonate treatments, and *Botrytis cinerea* infection (Li *et al.*, 2008).

Mammalian *ACBPs* are expressed (and the resulting proteins are produced) in a wide range of tissues, but, just like in plants, each ACBP is more specific to a certain organ or tissue type. As it was mentioned before, small cytosolic ACBPs in mammals are divided into three subcategories (isoforms), based on the primary tissue of expression. *L-ACBP* is highly expressed in liver, epithelial tissues and

secretory cells; *t-ACBP* expression is found mainly in testis, and *b-ACBP* was found to be highly expressed in brain (Faergeman *et al.*, 2007). Overall, *ACBPs* appear to be highly expressed in the cells with the high metabolism levels or secretory function (hepatocytes, steroid producing cells of the adrenal cortex and testis, cells in islets of the pancreas, tubular epithelial cells of the kidney, small intestinal epithelial cells) (Gossett *et al.*, 1996).

ACBP expression in mammals is modulated by hormones and feeding status via transcription factors such as sterol regulatory elements-binding protein 1 (SREBP-1) and peroxisome proliferator-activated receptors (PPAR) (Nitz *et al.*, 2005). Feeding rats a high-fat diet resulted in increased ACBP levels in liver, kidney and heart (Bhuiyan J. *et al.*, 1995). In hepatocytes, SREBP-1c is the main regulator of ACBP in response to changes in insulin levels during fasting/refeeding, while PPARα counteracts this effect by stimulating *ACBP* expression during fasting (Neess *et al.*, 2006).

2.2.5. Subcellular localization of ACBP

Cytosolic localization of small 10 kDa ACBP was first proposed by Hills *et al.* (1994) based on the absence of a signal peptide in the protein sequence (Table 2.2). Cytosolic localization of the *A. thaliana* 10 kDa ACBP was confirmed by analysis of expressed autofluorescence-tagged ACBP6 and western-blot analysis of subcellular fractions using ACBP6-specific antibodies (Chen *et al.*, 2008).

ACBP1 and ACBP2 were identified as membrane associated proteins, based on the presence of the transmembrane domain in aa sequence and co-localization with the subcellular membrane fraction (Chye, 1998; Li and Chye, 2003).

ACBP1-GFP and ACBP2-GFP fusion proteins transiently produced in onion epidermal cells co-localized with the plasma membrane (Li and Chye, 2003). Transport of ACBP1 and ACBP2 to the plasma membrane was proposed to be mediated by vesicles that are present in the subcellular fraction of large particles.

ACBP3 was identified as the only extracellular ACBP family member by the transient expression of the protein with DsRed-fusion in tobacco BY-2 cells and onion epidermal cells (Leung *et al.*, 2006).

Cytosolic localization of the ACBP4 and ACBP5 was demonstrated by confocal microscopy of autofluorescence-tagged proteins, expressed transiently in onion epidermal cells and in transgenic *Arabidopsis* (Xiao *et al.*, 2008b). ACBP4 was localized predominantly to the cytosol, but also to the periphery of the nucleus, perhaps as a result of the protein-protein interaction with AtEBP (Li *et al.*, 2008).

Recent studies with animal recombinant ACBPs suggest that the protein can interact/ associate with the intracellular membranes. Fluorescently labelled bovine liver ACBP targeted to the ER and Golgi in a ligand-binding-dependent manner in HeLa and bovine mammary gland epithelial cell lines (Hansen *et al.*, 2008). An ACBP variant unable to bind acyl-CoA no longer associated with the ER and became segregated from the Golgi. Mouse recombinant ACBP interacted with anionic phospholipid-rich, highly curved membranes (Chao H. *et al.*, 2002). Based on the computationally modeled interaction of HgACBP (armadillo Harderian gland) with anionic and neutral membranes, the long range electrostatic

forces were identified as the first step in deciphering the mode of interaction between ACBPs and membranes (Vallejo *et al.*, 2009).

2.2.6. Protein structure of ACBP

The primary structure of the small cytosolic ACBP shares a high degree of similarity between species (Figure 2.3). *A. thaliana* and *B. napus* ACBP are 92 amino acids long and have 83.7% identical amino acids. Plant small cytosolic ACBP sequences are approximately 70% similar, and share about 40% sequence identity with the animal ACBPs. The residues corresponding to Phe-7, Leu-27, Tyr-30, Lys-34, Ala-36, Gly-39, Pro-46, Lys-56, Trp-57, Trp-60, Ala-71 and Tyr-75 in *A. thaliana* ACBP are directly involved in ligand binding and are conserved across species. The ligand binding site of ACBP can be divided into three subsites: one for the 3'-phosphate, one for the adenine ring, and one for the acyl part of the ligand (Kragelund *et al.*, 1999). The 3'-phosphate group of the CoA part contributes approximately 40% of the total binding energy by interacting with ACBP through a network of two salt bridges to residues Lys-34 and Lys-56, and a hydrogen bond to Tyr-30 (Faergeman *et al.*, 2007). The aromatic rings of Tyr-33, Tyr-75 and Phe-7 structurally support the adenine ring through non-polar interactions. Single amino acid substitution of Tyr-30, Lys-34, Lys-56 and Tyr-75 in ACBP to Ala resulted in the mutant forms less effective in acyl-CoA binding than wild-type ACBP (Chye *et al.*, 2000). The non-polar ω-end of the acyl chain makes several van der Waals contacts to the non-polar side chains of Leu-27 and Ala-55.

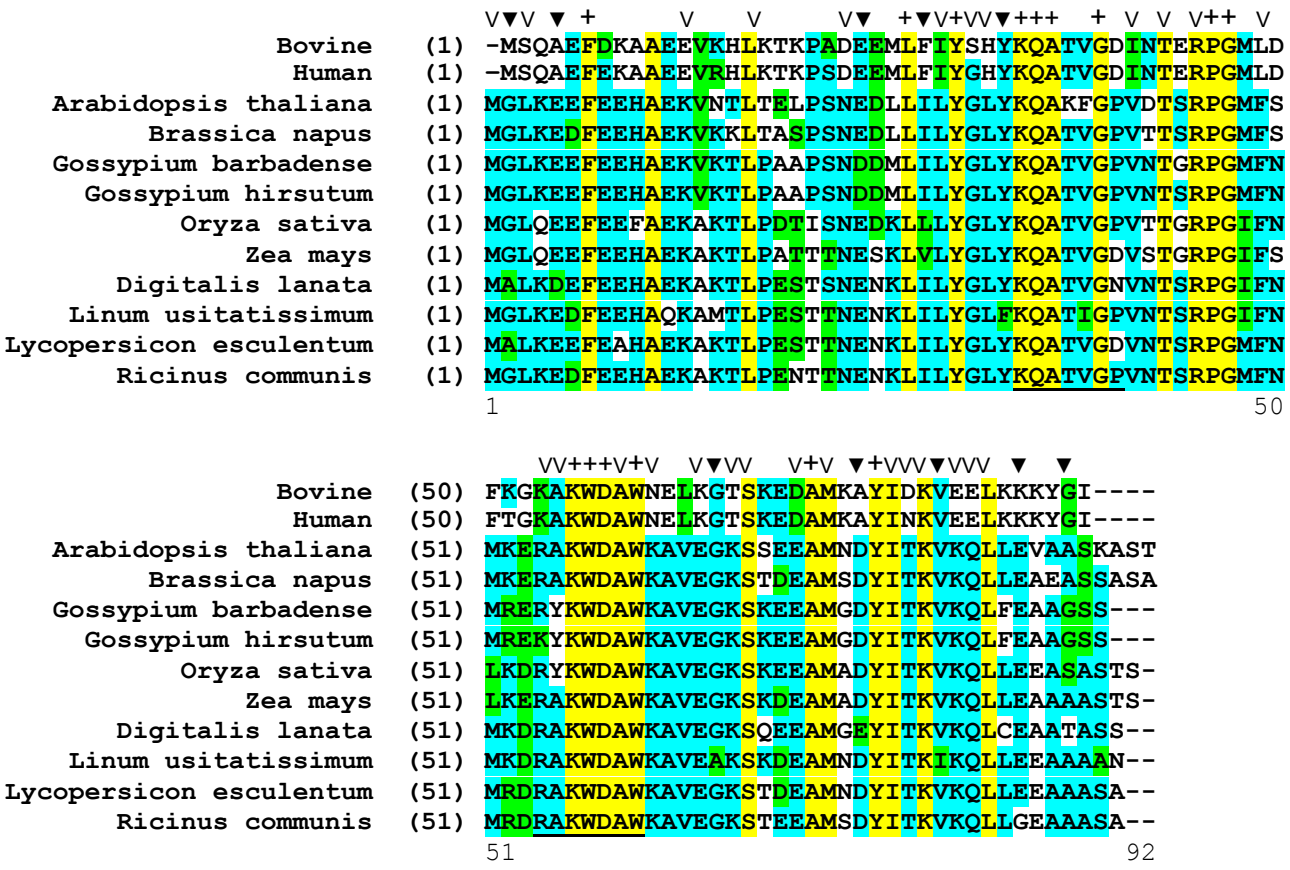


Figure 2.3. Amino acid sequence alignment of the cytosolic 10 kDa ACBP from plant and animal species. Plus sign (+) indicates amino acids conserved in all ACBPs; an open arrow (V) indicates amino acid residues conserved in plant ACBPs; a filled arrow (▼) represents amino acid residues proposed to be conserved in plant ACBPs by Engeseth *et al.* (1996), but obviously displaying a certain degree of variation. The underlined sequences correspond to the acyl-CoA-binding and the coenzyme A head group-binding sites, respectively.

The tertiary structure of ACBP is represented by four alpha helixes, designated A1 through A4 that run in an 'up-down-down-up' direction (Kragelund *et al.*, 1993) (Figure 2.4a). Crystal structure of the ACBP: acyl-CoA complex has been described for human, bovine and *Plasmodium falciparum* ACBPs (Kragelund *et al.*, 1993; van Aalten *et al.*, 2001; Taskinen *et al.*, 2007). The four alpha helices held together by hydrophobic interactions form a three-dimensional structure with the appearance of a "shallow bowl" or groove (Figure 2.4b). The ω-end of the acyl-chain is anchored in a hydrophobic cleft between helices A2 and A3 in the bottom of the binding site, while the CoA part of the ligand protects the acyl chain from interactions with the solvent (Faergeman *et al.*, 2007). Overall, the structure of the bound ligand seems to be organized in the binding site to protect the lipophilic acyl part from the polar environment. *A. thaliana* ACBP2 acyl-CoA binding domain has been proposed to consist of three helices instead of the classic four-helix structure described for bovine ACBP (Leung *et al.*, 2006).

An overhang loop connecting helices A2 and A3, which is not a part of the active binding site, has been proposed to play a role in initial capture of the ligand (Vallejo *et al.*, 2008). The amino acid sequence of the loop (Met46-Leu47-Phe49 in human and bovine ACBPs), although not conserved throughout phyla, retains the hydrophobicity (Figure 2.3). These residues, hydrophobic Met46 and Leu47, and charged residues Lys18 and Lys50 have been also proposed to play a role in the ACBP-membrane interaction and acyl-CoA extraction (Vallejo *et al.*, 2009).

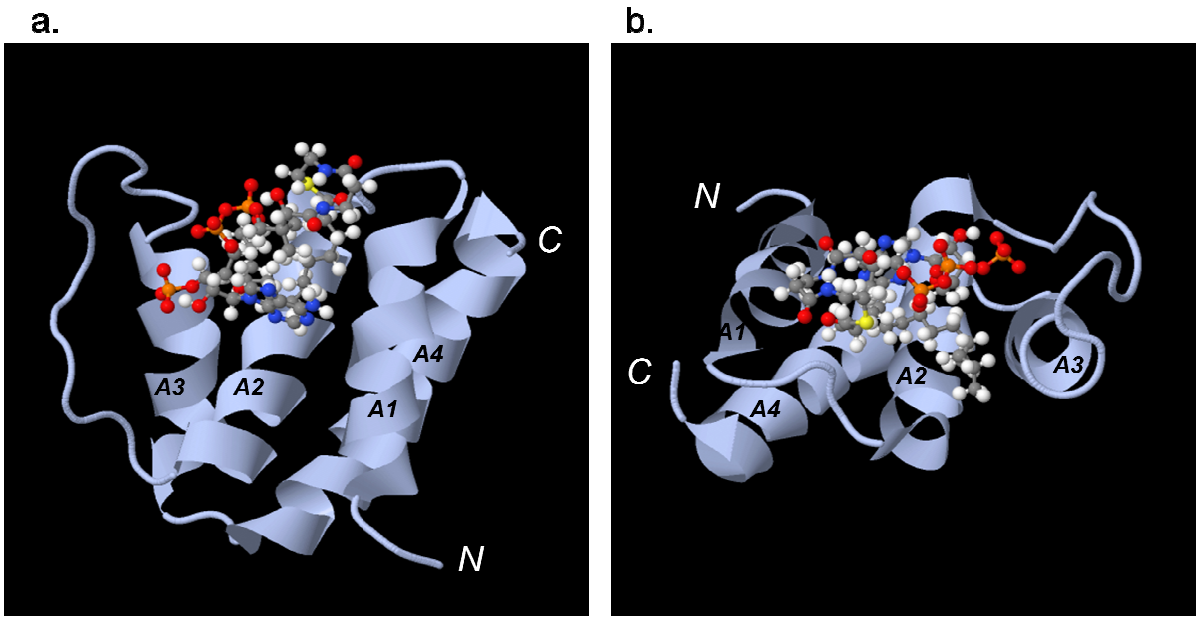


Figure 2.4. Image of the three-dimensional structure of the bovine ACBP complex with palmitoyl-CoA a. view from the front; b. view from the top (RCSB Protein Data Bank. www.rcsb.org/pdb). (Color designation for the parts of palmitoyl-CoA: grey – C, white – H, red – O, orange – P, yellow – S, blue – N).

ACBP is not expected to undergo any post-translational modifications. A phosphorylation-site, however, was detected in rat liver ACBP on Ser1 in the N-terminus and proposed to be involved in cellular signalling during apoptosis (Solstad *et al.*, 2008).

Most of the ACBPs described in *A. thaliana*, in addition to the acyl-CoA binding domain described above, contain other sequences that affect cellular localization and physiological effects of the protein (Table 2.2). The membrane bound ACBP1 and ACBP2 have a hydrophobic domain at the N'-terminus (Ile11–Phe31 and Val11–Val29, respectively), which is responsible for the membrane targeting of these proteins (Li and Chye, 2003). Five ankyrin repeats at the C'-terminus were also identified in ACBP1 and ACBP2 sequences immediately downstream from the acyl-CoA binding domain (Chye *et al.*, 2000). ACBP3 was predicted to contain a cleavable N'-terminal signal sequence (amino acids 1-26), which is likely responsible for the extracellular targeting of this ACBP member (Leung *et al.*, 2006). ACBP4 and ACBP5 are distinct from other *A. thaliana* ACBP members by the presence of five kelch motifs each. These are repetitive motifs of ca. 50 aa that, just like ankyrin repeats, constitute potential sites for protein-protein interactions (Li *et al.*, 2008).

Table 2.2. Characteristics of *A.thaliana* ACBP family proteins

ACBP	Amino acids	Mr (kDa)	pI	Binding domain	Additional domains	Subcellular localization	Binding preference for acyl group	Interacts with	Reference
ACBP (ACBP6)	92	10.4	4.91	3-87		Cytosol	18:3>18:2>18:1≈16:0	PC	1, 5, 14
ACBP1	338	37.5	4.32	94-180	transmem. domain; ankyrin repeats	PM and ER	20:4>18:2>18:3>18:1 >16:0	Pb(II)	2, 3, 4, 6, 13
ACBP2	354	38.5	4.22	104-190	transmem. domain; ankyrin repeats	PM and ER	18:2>18:3>20:4≈16:0 >18:1	AtEBP, AtFP6, Pb(II), Cd(II), Cu(II)	4, 6, 9, 10, 12
ACBP3	362	39.3	3.96	231-314	N' secretory signal	Extracellular	20:4>18:1>16:0		7,8
ACBP4	669	73.2	4.95	12-103	Five kelch motifs	Cytosol	18:1>16:0	AtEBP, PC	7, 11, 13, 15
ACBP5	648	71.0	5.86	22-104	Five kelch motifs	Cytosol	18:1>16:0	PC	7, 13, 15

References:

1 - Chen *et al.* (2008)
2 - Chye (1998)
3 - Chye *et al.* (1999)
4 - Chye *et al.* (2000)
5 - Engeseth *et al.* (1996)
6 - Gao *et al*. (2008)
7 - Leung *et al.* (2004)
8 - Leung *et al.* (2005)
9 - Li and Chye (2003)
10 - Li and Chye (2004)
11 - Li *et al.* (2008)
12 - Xiao *et al.* (2008a)
13 - Xiao *et al.* (2008b)
14 - Xiao and Chye (2009)
15 – Xiao *et al.* (2009)

2.2.7. Binding properties of ACBP

Numerous *in vitro* studies demonstrated acyl-CoA binding to a single binding site on ACBP (Reviewed in Gossett *et al.*, 1996). The binding is characterized by co-operativity, which seems to be a prerequisite for the high affinity binding. The binding site, described above, is arranged so that only acyl-CoA esters can bind strongly. Both the acyl-chain and the CoA part are important for the proper fit and strong interaction between ACBP and the ligand (Rosendal *et al.*, 1993). Thus, ACBP binds exclusively acyl-CoA esters and not free FAs, free CoA, ATP, NADH, NADPH, GABA, palmitoyl carnitine, or cholesterol. The binding resembles that of a "lock-and-key" model, which was confirmed by the fact that the protein structure underwent very little conformational change upon the ligand binding (Kragelund *et al.*, 1993).

Early binding studies of ACBP were performed using the Lipidex-1000 binding assay, which was originally developed to determine binding stoichiometry and dissociation constants for ligands binding to fatty-acid-binding protein (Glatz and Veerkamp, 1983). This simple method is based on the ability of the Lipidex-1000 beads to effectively remove all unbound ligand without interfering with ligand binding to the protein. ACBP, however, have been shown to extract acyl-CoA esters bound to Lipidex due to its high affinity to acyl-CoA (Rosendal *et al.*, 1993). Therefore, the Lipidex-1000 binding assay is considered unsuitable for determining true binding constants, but rather serves as an indicator of relative binding affinity. Titration calorimetry is another method for performing binding assays which allows the accurate determination of binding

constants and thermodynamic parameters (Miller and Cistola, 1993). Binding affinities for acyl-CoAs were estimated to be in the low micromolar range (Kd = 0.1–20μM) for plant and animal ACBP using the Lipidex-1000 binding assay (Rasmussen *et al.*, 1990; Gossett *et al.*, 1996; Leung *et al.*, 2006). Dissociation constants obtained from the microcalorimetry measurements, on the other hand, were at least one order of magnitude lower than values estimated using the Lipidex-1000 assay (Faergeman *et al.*, 1996; Pacovsky, 1996). The binding affinity of acyl-CoA esters for ACBP has been shown to be strongly dependent on the length of the acyl chain with a clear preference for acyl-CoA esters containing more than eight carbon atoms (Faergeman *et al.*, 1996). Acyl-CoA with a chain-length of C12-C22 were bound strongly by recombinant AthACBP with C18 and C20 being the most preferable length of the ligand (Pacovsky, 1996). Acyl-CoAs of the same number of carbons in acyl chain still can be differentiated by *A. thaliana* ACBPs based on the number of double bonds (Pacovsky, 1996; Leung *et al.*, 2004 and 2006; Gao *et al.*, 2008). No correlation between the number of double bonds in the acyl-chain and the binding affinity, however, was observed for the bovine rACBP (Rosendal *et al.*, 1993). Different ligand specificities in combination with different cellular localizations of the *A. thaliana* ACBPs suggest that these proteins may have distinct specialized functions (Table 2.2).

The binding site of the small cytosolic ACBPs can accommodate only one molecule of the ligand. The binding stoichiometry values calculated for hexadecanoyl-CoA binding by bovine and rat ACBPs were close to 1 mol/mol (Rasmussen *et al.*, 1990; Frolov and Schroeder F., 1998). Recently a different

binding mode was observed in the crystal structures of the ligand-bound human cytosolic liver ACBP, where myristoyl-CoA was bound by two ACBP molecules (Taskinen *et al.*, 2007). The 3'-phosphate-AMP moiety of the ligand was bound in the binding pocket of one ACBP molecule and the acyl chain was bound in the pocket of the other ACBP molecule (Taskinen *et al.*, 2007). This binding mode might be relevant for situations when acyl-CoA is extracted or donated by ACBP.

It was demonstrated that ACBP can interact with molecules other than the protein's primary ligand. Small soluble ACBP from *Arabidopsis* has been shown to associate with PC but not PA or LPC in the filter-binding assay (Chen *et al.*, 2008). Binding affinity and stoichiometry of this interaction have not been determined yet.

A. thaliana ACBP1 and ACBP2 were able to bind Pb(II) *in vitro* (Xiao *et al.*, 2008a; Gao *et al.*, 2008). ACBP2 could also bind Cd(II), and Cu(II) (Gao *et al.*, 2008). The membrane-bound ACBPs were proposed to be involved in transport and accumulation of these metals in roots. This notion is supported by the fact that *A. thaliana* overexpressing ACBP1 or ACBP2 were more tolerant to Pb(II) and Cd(II), respectively, than wild-type (Xiao *et al.*, 2008a; Gao *et al.*, 2008).

ACBP2 was shown to interact with the *A. thaliana* ethylene-responsive element-binding protien (AtEBT) and farnesylated protein 6 (AtFP6) via ankyrin repeats (Li and Chye, 2004; Gao *et al.*, 2008). The protein-protein interaction was abolished when the ankyrin repeats were removed from ACBP molecule. AtEBP was also identified as an interaction partner for ACBP4 (Li *et al.*, 2008). AtEBP is

expressed mainly in response to biological or physical stress, such as pathogen attack, hormone treatment, drought, cold, or cadmium treatment (Büttner and Singh, 1997; Li *et al.*, 2008). Thus, ACBP2 and ACBP4 interaction with AtEBT may have a physiological role in responses to biotic and abiotic factors (Büttner and Singh, 1997; Gao *et al.*, 2008). The fact that *ACBP2* and *ACBP4* are highly expressed in roots, the main port of entry for minerals and pathogens, also supports the proposed role in protection mechanisms for these proteins.

2.2.8. Biochemical functions of ACBP

ACBP binds acyl-CoA esters with high affinity and most of its functions derive from this very ability. *In vitro* and *in vivo* experiments suggest that ACBP is involved in multiple cellular tasks including maintenance, protection and transport of the acyl-CoA pool, regulation of the enzyme activities and gene expression, and membrane protection from the acyl-CoAs.

Acyl-CoA pool maintenance. ACBP is a major factor in the formation of the cytosolic acyl-CoA pool. ACBP regulates the concentration of free acyl-CoA in the cytosol, keeping it at low nanomolar levels (Knudsen J. *et al.*, 1999). Depletion or overexpression of ACBP in *S. cerevisiae* resulted in changes in the acyl-CoA pool size and composition (Mandrup S. *et al.*, 1993; Knudsen J. *et al.*, 1994; Gaigg B. *et al.*, 2001). Expression of bovine ACBP in yeast resulted in a significant increase in the relative contribution of 16:0 to the total pool of acyl-CoA, while the relative content of 18:0 and 18:1 were markedly decreased (Knudsen J. *et al.*, 1994). In Acb1p depleted yeast cells, the level of 18:0 increased 3.5-fold in acyl-CoA pool, whereas that of 14:0, 16:1 and 18:1 were

reduced by 2- to 2.5-fold (Gaigg B. *et al.*, 2001). This suggests that ACBP availability may play a role in termination of FA biosynthesis and in transport or presentation of FA for modifications.

In transgenic mice overexpressing ACBP, the liver acyl-CoA pool size increased by 69% preferentially in saturated and polyunsaturated, but not monounsaturated acyl-CoA (Huang *et al.*, 2005). Overexpression of ACBP in the transgenic rat resulted in elevated acyl-CoA tissue levels, mainly due to an increase in C16 and C20 levels (Oikari *et al.*, 2008).

Protection of the Acyl-CoA pool from hydrolysis. Both cytoplasm and microsomal membranes contain high levels of acyl-CoA hydrolases. ACBP is effective in protecting acyl-CoA from hydrolysis by microsomal membranes (Rasmussen *et al.*, 1993; Engeseth *et al.*, 1996). Other proteins able to bind acyl-CoA like bovine serum albumin (BSA) and liver fatty acid binding protein (l-FABP) were not nearly as effective as ACBP in protecting acyl-CoA.

Intra- and extracellular transport of Acyl-CoA. ACBP can sequester acyl-CoA esters and facilitate their transport to the catalytic sites of selected enzymes. ACBP has been shown to transport acyl-CoA from immobilized membranes to mitochondria or microsomes in suspension for FA β-oxidation or glycerolipid synthesis, respectively (Rasmussen *et al.*, 1994). Dramatic reduction of C26:0 in the acyl-CoA pool of Acb1p-depleted yeast suggested ACBP may be involved in transport of *de novo* synthesized acyl-CoA from FAS to FA elongation, or between the elongation steps (Gaigg B. *et al.*, 2001).

ACBP was identified as a major phloem sap protein in rice (*Oryza sativa* L.), cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita maxima* Duch.) and other angiosperms (Walz *et al.*, 2004; Suzui *et al.*, 2006). Despite the degradation of several major organelles, mature sieve elements contain metabolically active mitochondria (Sjolund, 1997), and significant amounts of ATP have been detected in phloem sap (Ohshima *et al.*, 1990). Thus, it has been proposed that ACBP may transport via plasmodesmata acyl-CoAs synthesized in plastids of the companion cells to the sieve elements for β-oxidation in mitochondria. ACBP may also mediate the translocation of acyl-CoA from the source-organ to the sink-organ which needs substrates for membrane synthesis.

Protection of the cellular membranes from acyl-CoAs. As it has been mentioned before, acyl-CoAs can partition into the lipid bilayer of the cellular membranes due to their amphipathic nature. This, in turn, can cause significant alterations in the physical properties of the membrane and affect the activity of membrane-bound enzymes. ACBP has been proposed to play a role in protecting membranes against damage from the detergent activity of acyl-CoAs. ACBP was able to extract acyl-CoA from PC membranes immobilized on nitrocellulose (Rasmussen *et al.*, 1994). Bovine recombinant ACBP was able to extract acyl-CoA molecules from membranes by direct interaction and binding to acyl-CoA molecules embedded in the membrane (Cohen Simonsen *et al.*, 2003). BSA was unable to fully extract membrane-bound acyl-CoA.

Regulation of the enzyme activities. ACBP can modulate the activity of the enzymes involved in acyl-CoA synthesis and utilization by regulating acyl-CoA

concentration and availability. Even low concentrations of the free intracellular acyl-CoAs strongly inhibit ACS activity. ACBP can effectively oppose this product feedback inhibition of the ACS by sequestration of the synthesized acyl-CoA esters. Bovine liver ACBP was first discovered to induce the synthesis of medium-chain acyl-CoA esters by goat mammary gland fatty acid synthetase *in vitro* (Mogensen *et al.*, 1987). Recombinant bovine ACBP also stimulated the synthesis of acyl-CoA esters by mitochondria from rat liver (Rasmussen *et al.*, 1993).

The low concentration of intracellular free acyl-CoA *in vivo* is also crucial for maintaining the activity of important enzymes such as acyl-CoA carboxylase (ACCase) and adenine nucleotide translocase (ANT). Recombinant bovine ACBP was found to have a strong attenuating effect on the acyl-CoA inhibition of both ACCase and mitochondrial ANT *in vitro* (Rasmussen *et al.*, 1993).

The presence of ACBP in *B. napus* embryo cells allows for regulation of the glucose-6-phosphate transporter, which is very sensitive to small changes in acyl-CoA concentration (Fox *et al.*, 2000b). While keeping free acyl-CoA concentrations low, ACBP maintains relatively high concentrations (10-20 μM) of acyl-CoAs bound to ACBP available for the acyltransferases in the endoplasmic reticulum.

It has been suggested that the ratio of acyl-CoA and ACBP is important in regulating TAG and phospholipid synthesis (Rasmussen *et al.*, 1993). A number of *in vitro* experiments have shown that recombinant plant or animal ACBP can either stimulate or inhibit the activity of Kennedy pathway acyltransferases

(GPAT, LPAAT), and phospholipid and cholesterol ester biosynthesis (LPLAT, ACAT) depending on substrate concentrations and incubation conditions (Fyrst *et al.*, 1995); Jolly *et al.*, 2000; Kannan *et al.*, 2003; Chao *et al.*, 2003). The effect of ACBP on the acyltransferase activities *in vitro* will be discussed in more detail in Chapter 4. Here we will only review the effects of up- or down-regulation of ACBP expression on lipid biosynthesis *in vivo*.

Down-regulation of *ACBP* in 3T3-L1 adipocytes resulted in a decrease in lipid accumulation and degree of cell differentiation (Mandrup *et al.*, 1998). Deletion of *Acbp* in mouse resulted in FA metabolism abnormalities, with hair lipid profiles showing altered levels of TAG and co-migrating lipids (Lee *et al.*, 2007). *ACBP* overexpression in mice resulted in a nearly 2-fold increase in PL and TAG levels in liver, and in a higher relative content of 18:1 in liver PLs (Huang *et al.*, 2005). Enzymatic activity of liver microsomal GPAT increased 4-fold in transgenic mice.

A. thaliana knock-out mutants of the large cytosolic ACBP4 showed decreases in membrane lipids (digalactosyldiacylglycerol, monogalactosyldiacylglycerol, phosphatidylcholine, phophatidylethanolamine and phosphatidylinositol), suggesting that ACBP4 plays a role in the biosynthesis of galactolipids and phospholipids (Xiao *et al.*, 2008b). Down-regulation of the membrane-bound ACBP2, on the other hand, did not affect the levels of the predominant lipid classes (DGDG, MGDG, PC, PE, PI, PA, phosphatidylserine and phosphatidylglycerol), but resulted in some significant changes in acyl chain

composition of the phospholipid species (Mishra *et al.*, 2004; Kojima *et al.*, 2007).

Membrane biogenesis. ACBPs have been proposed to be involved in vesicular trafficking, organelle biogenesis and membrane assembly mainly based on the phenotype of *Saccharomyces cerevisiae* depleted of Acb1p (Gaigg B. *et al.*, 2001; Faergeman *et al.*, 2004). The mutant yeast cells displayed multilobed vacuoles, invaginations and multi-layered structures of the PM, and a large number of vesicles of variable sizes, autophagocytotic like bodies and membrane fragments. Vacuoles isolated from Acb1p-depleted cells were unable to undergo homotypic vacuole fusion *in vitro*. Dramatic reduction in the content of ceramides in whole-cell lipids and in vacuoles isolated from Acb1p-depleted cells might have been the reason for such dramatic abnormalities in the phenotype (Faergeman *et al.*, 2004). *A. thaliana* membrane-bound ACBP1 was proposed to be involved in intracellular lipid transfer from the ER, via vesicles, to the PM (Chye *et al.*, 1999).

Regulation of the gene expression. ACBPs have been shown to affect the levels and/or activity of some transcription factors in mammals. Down-regulation of *ACBP* in 3T3-L1 adipocytes resulted in a significant delay in the induction of the adipogenic transcription factors (C/EBPα and PPARγ) (Mandrup *et al.*, 1998). It was proposed that abrogation of *ACBP* expression in 3T3-L1 cells might have prevented proper synthesis and/or handling of the ligands necessary for PPARγ activation. Overexpression of *ACBP* in transgenic rats resulted in down-regulation of the transcription factors SREBP-1 and PPARs (Oikari *et al.*, 2008). Liver

ACBP has been shown to form a functional complex with HNF-4α *in vivo*. Overexpression of *l-ACBP* in COS-7 or rat hepatoma cells was found to enhance HNF-4α mediated transcriptional activity (Petrescu *et al.*, 2003). Depletion of the yeast Acb1p resulted in a 5-fold increase in the Δ9-desaturase *OLE1* mRNA level (Gaigg B. *et al.*, 2001).

Protection from and response to environmental factors. ACBP may be involved in the synthesis of the external lipid-based protective layers in plants and in animals. High levels of expression of the membrane-bound ACBP identified in *Agave americana* leaf epidermis suggested an active participation of the protein in the transport of acyl-CoA esters constituent of cuticular components as waxes, cutin and/or cutan monomers, from the epidermal cell to the outside (Guerrero *et al.*, 2006). ACBP was proposed to be important for proper hair and skin development and maintenance in the mouse, based on the observed abnormalities in the appearance of the *Acbp* depleted mouse (Lee *et al.*, 2007).

Arabidopsis knock-out of the small cytosolic ACBP demonstrated increased sensitivity towards freezing, while plants overexpressing ACBP has an enhanced freezing tolerance, which was mediated by the phospholipase Dδ (PLDδ) pathways (Chen *et al.*, 2008). Membrane-bound ACBPs in *Arabidopsis* appear to be involved in the protective mechanisms against heavy metals found in soil. Plants over-expressing ACBP1 or ACBP2 showed enhanced tolerance to Pb(II) and Cd(II) in the growth medium, respectively, compared to WT (Xiao *et al.*, 2008a; Gao *et al.*, 2008). ACBP2 was also proposed to play a role in PL repair following lipid peroxidation under heavy metal stress at the PM (Gao *et al.*,

2008). Three members of the Arabidopsis ACBP family (ACBP1, ACBP3 and ACBP4) have been suggested to play a role in lipid-mediated signalling during pathogen infection (Leung *et al.*, 2004 and 2006; Li *et al.*, 2008).

Protein acylation. L-ACBP strongly suppresses non-enzymatic thiolation of cysteinyl-containing peptides. In contrast to non-enzymatic acylation, enzymatic protein acylation remained active in the presence of physiological concentrations of l-ACBP and acyl-CoA, suggesting that l-ACBP can donate acyl-CoA for protein thioacylation (Dunphy *et al.*, 2000).

Insulin signalling. An increased concentration of free acyl-CoA has been proposed as factor involved in the development of insulin resistance. In the muscles from obese insulin resistent Zucker rats, the 90% increase in the concentration of acyl-CoA was not matched by the 30% increase in ACBP levels (Franch J. *et al.*, 2002). ACBP has been shown to inhibit glucose-stimulated insulin release from pancreatic islet β cells (Borboni *et al.*, 1991; Chen Z.-W. *et al.*, 1988).

The human *ACBP* was identified as a potential candidate gene for type 2 diabetes, since it plays a central role in determining the intracellular concentration of activated FAs. Two Caucasian study populations provided evidence that a minor allele of ACBP (single nucleotide polymorphisms) might be associated with reduced risk of type 2 diabetes (Fisher *et al.*, 2007).

2.3. Other lipid binding proteins

ACBP is certainly not the only small soluble protein capable of acyl-CoA binding. Nonspecific lipid transfer protein (nsLTP), sterol carrier protein-2 (SCP-2), fatty-acid binding protein (FABP) and serum albumin are other soluble proteins that can bind a wide range of lipid molecules, including acyl-CoA (Table 2.3). These proteins found in different phyla are involved in one or the other aspect of buffering and trafficking of lipids within and outside of the cell. Knowledge of the structure, functions and binding properties of these proteins is helpful in studying and understanding the physiological role of ACBP in the context of global lipid management throughout the organism.

Overall, the small lipid binding proteins mentioned above share a number of structural and functional similarities. Most of these protein classes are represented by multiple isoforms with distinct functions and expression patterns. Lipid binding proteins are ubiquitously expressed throughout the organism, with higher expression in tissues with the highest rates of lipid metabolism. All soluble lipid binding proteins share a common structural feature – a hydrophobic cavity, which directly interacts with the ligand and separates it from the aqueous exterior. It appears that each protein has one preferred type of ligand, which corresponds to the primary binding partner *in vivo*. At the same time, structural flexibility of the binding domains allows for a broad range of ligands to interact with the protein. Even though binding specificities of the lipid binding proteins expressed in the same tissue appear to overlap, a certain degree of specialization in binding different acyl-CoA species is observed for these proteins.

Table 2.3. Characteristics of the soluble lipid binding proteins.

Protein	Mr (kDa)	Representation among Phyla	Spatial expression	Subcellular localization	Tertiary structure	Binding specificity	Functions	Reference
ACBP	10	Eukaryotes, 11 eubacteria	All tissues (tissue-specific distribution of the isoforms in animals)	cytosol	4 α-helices held by hydrophobic interactions	Acyl-CoA	Acyl-CoA pool maintenance and protection; acyl-CoA transport; regulation of enzyme activities and gene expression; membrane biogenesis	10, 11, 14, 20, 30
LTP	9	Plant Kingdom	All tissues (higher in peripheral cell layers)	extracellular	4 α-helices held by 4 disulfide bridges	FA, acyl-CoA, PL, LPL, glycolipids	Role in defense mechanisms against phytopathogens. Proposed roles in cutin formation, embryogenesis, symbiosis	8, 15, 17, 19, 21, 25, 28
SCP-2	14	Eukaryotes, Bacteria, Archaea	All tissues (higher in flowers and seeds in plants; liver, intestine, testis, ovary in animals)	Peroxisome (in animals also found in cytosol and mitochondria)	Five stranded β-sheet and 4 or 5 α-helices	Cholesterol, sphingolipids, PL, FA, acyl-CoA	In animals stimulates cholesterol esterification and transport, oxidation of branched-side chain lipids; in plants is important for the seed and seedling metabolism	4, 9,13, 16, 18, 22, 26, 29, 31
FABP	14-15	Animal Kingdom	All tissues (tissue-specific distribution of the isoforms)	Cytosol, near ER (low levels in mitochondria and nucleus)	10 β-strands, organized into 2 β-sheets , has a helix-turn-helix motif	FA, LPL, prostaglandins, cholesterol, bile acids, acyl-CoA, bile acids	Cellular uptake and transport of FA, targeting of FA to specific metabolic pathways, regulation of gene expression and cell growth.	1, 5, 6, 16, 23, 27, 32
Serum albumin	66.3	Mammals	Is synthesized in liver, found mainly in blood plasma	extracellular	α-helical segments organized into 3 domains by disulfide bonds	FA, acyl-CoA, thyroid hormones, many drugs, Ca^{2+} ions	Transport of FA; maintenance of colloidal blood pressure, bodily detoxification	2, 3, 7, 12, 24

References for **Table 2.3**:

1 - Boord *et al*. (2002)
2 - Boylan and Hamilton (1992)
3 - Brown and Shockley (1982)
4 - Choinowski *et al*. (2000)
5 - Chmurzynska (2006)
6 - Coe and Bernlohr (1998)
7 - Curry *et al*. (1999)
8 - Douliez *et al*. (2001)
9 - Edqvist *et al*. (2004)
10 - Færgeman *et al*. (2004)
11 - Færgeman *et al.* (2006)
12 - Fasano *et al*. (2005)
13 - Frolov *et al*. (1996)
14 - Gaigg *et al*. (2001)
15 - Garcia-Olmedo *et al*. (1995)
16 - Gossett *et al*. (1996)
17 - Guerbette *et al*. (1999)
18 - Haapalainen *et al.* (2001)
19 - Kader (1996)
20 - Kragelund *et al*. (1999)
21 - van Loon and van Strien (1999)
22 - Moncecchi *et al.* (1991)
23 - Paulussen and Veerkamp (1990)
24 - Richards *et al*. (1990)
25 - Salcedo *et al.* (2007)
26 - Schroeder *et al*. (2007)
27 - Storch and McDermott (2009)
28 - Tassin-Moindrot et al. (2000)
29 - Vahouny *et al*. (1987)
30 - Xiao and Chye (2009)
31 - Zheng *et al*. (2008)
32 - Zimmerman and Veerkamp (2002)

For example, recombinant human SCP-2 bound fatty acyl-CoAs with carbon chain lengths ranging from C10 to C18, with maximal interaction for C12 - C16 (Frolov A. *et al.*, 1996). In contrast, animal ACBPs bind acyl-CoA esters over a wider range than SCP-2, with acyl chain length ranging from 8-22 carbons and highest affinity for 14-22-carbon chain length acyl-CoA (Knudsen *et al.*, 1989; Rosendal *et al.*, 1993). Another physiological similarity shared by the lipid binding proteins is their role in solubilization, buffering and transport of the lipid ligand. More specialized functions can also be taken up by specific isoforms based on the metabolic demands of a certain tissue.

Bovine serum albumin (BSA) is routinely utilized *in vitro* to prevent the adverse detergent effects of acyl-CoA esters in enzyme assays (Richards *et al.*, 1990). BSA has been also shown *in vitro* to modulate the activity of acyltransferases involved in seed oil formation (Details in Chapter 4). *In vivo* BSA has also been shown to affect the biosynthesis of seed oil. *B. napus* transformed with cDNA encoding BSA exhibited a significant increase in 18:2 and α-18:3 content in mature seeds (Weselake *et al.*, 2003).

2.4. Summary

Acyl-CoA is a major intermediate of lipid metabolism in the living cell. In plants, FAs are synthesized in plastids and get activated to acyl-CoAs upon their transfer to the cytosol. Acyl-CoAs undergo further modifications (elongation, desaturation) outside the plastids on the membranes of the ER. Acyl exchange between acyl-CoA pool and membrane phospholipids appears to have an important role in defining the acyl-CoA composition. In the cell, acyl-CoAs are

utilized for biosynthesis of structural and storage lipids. They are also involved in regulation of enzyme activities, gene expression, protein modifications and membrane fusions. In developing seeds, acyl-CoAs are used in the biosynthesis of TAG, a major component of seed oil. TAG is synthesized on the ER and accumulates in small organelles, known as oil bodies, which are enclosed by a half-unit membrane. Oleosin is a major protein component of this membrane. It prevents oil bodies from coalescence during seed desiccation and facilitates the utilization of seed oil during seed germination and seedling establishment.

The intracellular concentrations of acyl-CoA require well regulated control in order to prevent micelle formation and acyl-CoA partitioning into membranes, and the inhibition of certain enzymes and transporters. Another aspect of acyl-CoA pool maintenance is the compartmentalization of the lipid metabolism, which requires a directed intracellular transport of acyl-CoA in the aqueous environment. ACBP is recognized as the main acyl-CoA pool former and transporter ubiquitously found in all eukaryotic organisms studied. ACBP is a small ~10 kDa cytosolic protein with a high degree of conservation in amino acid sequence among species. The binding domain of ACBP consists of four α-helices organized into a “shallow bowl”-like structure with a hydrophobic cavity that can specifically accommodate an acyl-CoA molecule. The protein binds acyl-CoA esters with high affinity and can differentiate among different acyl-CoA species on a basis of the acyl-chain length and number of double bonds. In addition to the acyl-CoA binding domain, some ACBPs found in plants and in animals contain other domains involved in protein targeting and subcellular localization, or

mediate protein-protein interactions. Within the cell, ACBP maintains the acyl-CoA pool and protects it from the hydrolysis. Buffering of the acyl-CoAs by ACBP also protects cellular membranes and enzymatic activities from the adverse effects of high concentrations of free acyl-CoA. ACBP also plays roles in intra- and extracellular transport of acyl-CoA, regulation of enzyme activities and gene expression, response mechanisms to environmental factors and other processes which acyl-CoAs are involved in. The main focus of the following chapters will be on further understanding of the physiological role of small cytosolic ACBP in seed oil biosynthesis. Knowledge of the physiological properties of other lipid binding proteins is useful in planning experiments and interpreting experimental results in the path to uncovering new functions of ACBP.

3. Overexpression of *B. napus* 10 kDa ACBP in *A. thaliana* and *B. napus* developing seeds

3.1 Introduction

The acyl-CoA binding proteins (ACBP) and acyl-CoA levels have been shown to be very similar in *B. napus* embryos (Fox *et al.*, 2000a). Due to the high affinity of ACBP to the ligand, most of the acyl-CoAs in the cytosol are expected to be associated with ACBPs. The ratio of ACBP to acyl-CoA changes throughout the development of the embryo, which can affect the activity of certain enzymes involved in seed oil biosynthesis (Fox *et al.*, 2000a; Johnson *et al.*, 2000). We proposed that by overexpressing small cytosolic ACBP in developing seed, we can change the ACBP: acyl-CoA ratio, which in turn might affect biochemical pathways of seed oil formation.

Evidence suggests that ACBP may have an important role in triacylglycerol (TAG) accumulation in developing seeds. *B. napus* ACBP, a small cytosolic protein of 92 amino acids, was expressed at higher levels in developing embryos and flowers compared to other parts of the plant (Hills *et al.*, 1994). More careful examination of *ACBP* expression in developing seeds revealed that the highest concentration of the protein coincided with the peak of TAG accumulation (Engeseth *et al.*, 1996). The results of *in vitro* experiments showed that recombinant *B. napus* ACBP stimulated *sn*-glycerol-3-phosphate acyltransferase (GPAT) activity in a manner dependent on the ACBP: acyl-CoA ratio in the reaction mixture (Brown *et al.*, 1998).

Overexpression of ACBP in yeast and animals has been shown to increase the acyl-CoA pool size and rates of glycerolipid synthesis (Mandrup *et al.*, 1993b; Huang *et al.*, 2005). In transgenic mice overexpressing ACBP, the liver long-chain acyl-CoA pool size increased by 69% and liver microsomal GPAT activity increased almost 4-fold (Huang *et al.*, 2005). One of the few published results on *ACBP* overexpression in plants demonstrated that production of *A. thaliana* 10kDa ACBP in low and high erucic rapeseed varieties resulted in modifications in FA composition of seed oil in a manner dependent on the vector structure and genetic background of the transformed plants (Enikeev and Mishutina, 2005). Overexpression of *A. thaliana* 10 kDa ACBP using a 35S promoter resulted in enhanced freezing tolerance accompanied by an increase in phosphatidic acid (PA) and a decrease in phosphatidylcholine (PC) content in leaf tissue (Chen *et al.*, 2008). Overexpression of *A. thaliana* *ACBP1* and *ACBP2* encoding membrane-bound proteins enhanced plant tolerance to Pb(II)- and Cd(II)-induced stress, respectively (Xiao *et al.*, 2008a; Gao *et al.*, 2008). The physiological role of small cytosolic plant ACBPs in seed oil biosynthesis, however, remains unclear.

We have engineered *A. thaliana* to produce cytosolic 10 kDa ACBP from *B. napus* (BnACBP) in developing seeds. Eight constructs with different targeting sequences were produced. Two constructs were made for cytosolic production of BnACBP. Four out of eight constructs represent *BnACBP* fusions with *A. thaliana* oleosin gene variants. These constructs were designed to target expression of the acyl-CoA binding sites to the surface of the endoplasmic reticulum (ER), where

enzymes of the fatty acid (FA) modification and Kennedy pathway reside. Another two constructs were designed for BnACBP production and retention to the ER lumen. Our results demonstrate that overexpression of *ACBP* in developing seeds affects FA composition of the seed oil and acyl-CoA pool in developing seeds. Protein levels and sub-cellular localization of the recombinant BnACBP in transgenic *A. thaliana* seeds were also analyzed and compared between the constructs.

3.2 Experimental procedures

3.2.1 Molecular cloning of genetic constructs for *BnACBP* expression in developing seeds

cDNA encoding *B. napus* cytosolic ACBP was obtained from Picoscript (Houston, TX, USA) based on cDNA sequence available from the GenBank database (Accession number X77134). Restriction endonuclease sites were added to *B. napus* ACBP cDNA (*NcoI* and *XhoI* at the 5', and *HindIII* at the 3'-end) to facilitate molecular cloning of cDNA into pSBS binary vectors (SemBioSys Inc., Calgary, Canada) containing phaseolin promoter/ terminator expression system and the fusion partner genes. All binary vectors had an antibiotic resistance marker gene (Spectinomycin) for bacterial selection, and herbicide resistance gene (pat) within T-DNA for plant selection. Conventional cloning procedures were used for molecular cloning and bacterial transformation (Appendix 1).

Four molecular constructs were designed to encode BnACBP in-frame fusions with *A. thaliana* 18 kDa oleosin (GenBank accession number X62353.1): N' and C' terminal fusion, N'-fusion with oleosin variant 32 amino acids deletion

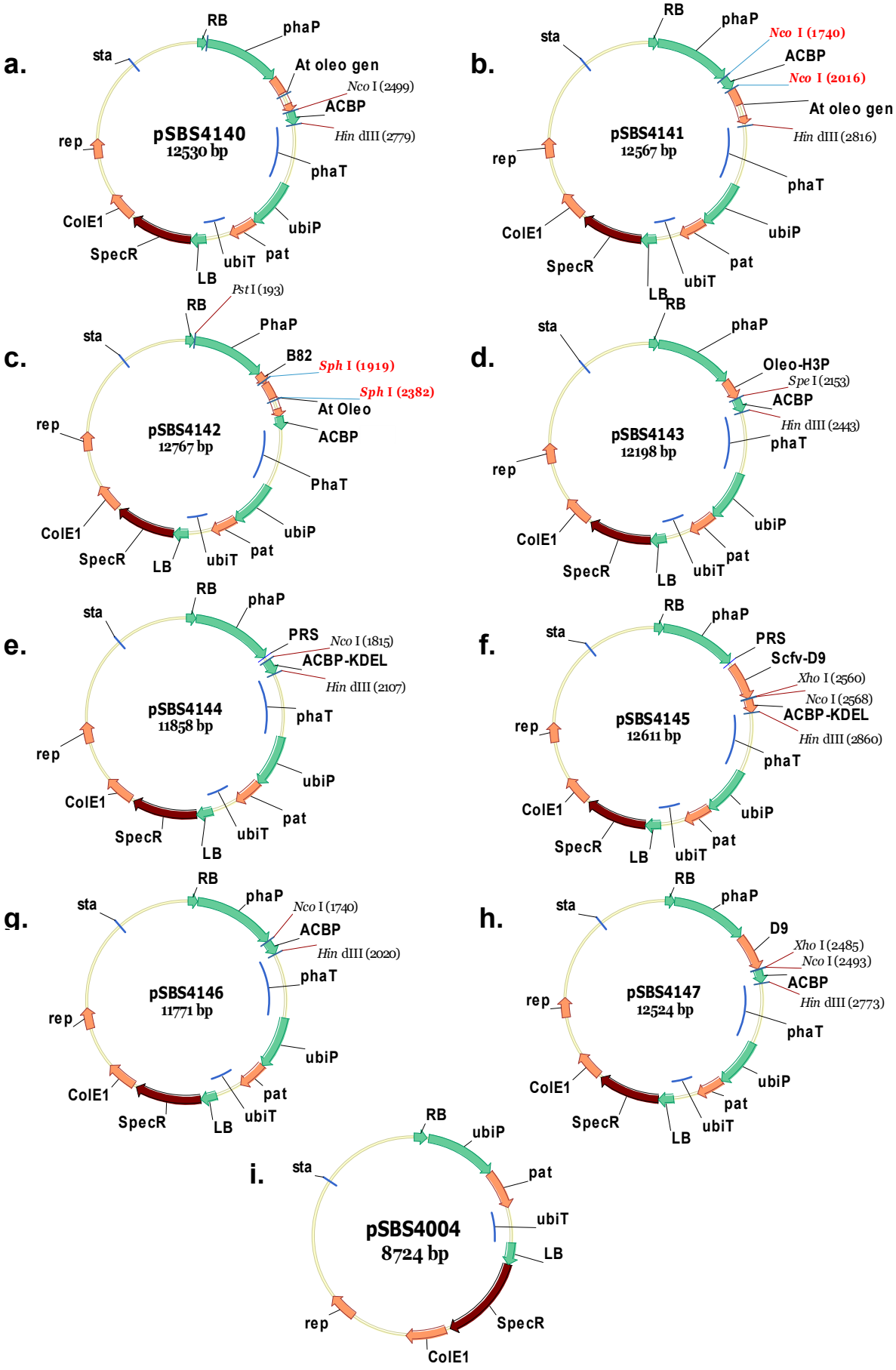
in the hydrophobic domain (OleoH3P), and C'-fusion with oleosin variant containing a 82 amino acid N'-terminal domain from the berberine bridge enzyme (GenBank accession number AF025430.1). These constructs named ACBP-Oleo, Oleo-ACBP, ACBP-OleoH3P and B82-Oleo-ACBP were cloned into the binary vectors, resulting in the final plasmids pSBS4140, pSBS4141, pSBS4142, and pSBS4143, respectively (Figure 3.1a-d).

Two constructs designed for cytosolic expression were ACBP and ACBP N'-terminal fusion with a single chain variable fragment of the antibody against *A. thaliana* 18 kDa oleosin (D9). ACBP and D9-ACBP constructs were cloned into the final vectors pSBS4146 and pSBS4147 (Figure 3.1g-h).

Two constructs with the luminal retention signal, ACBP-KDEL and D9-ACBP-KDEL, were cloned into the binary vector designed for ER expression of the transgene, resulting in the plasmids pSBS4144 and pSBS4145 (Figure 3.1e-f).

All eight genetic constructs were sequenced to confirm the in-frame fusion and a correct nucleotide sequence. A pSBS4004 vector harbouring only selection marker (pat) in T-DNA was used as a Null Vector (Figure 3.1i). Details of the cloning strategies and sequences of the BnACBP molecular constructs for seed specific expression in *A. thaliana* are provided in the Appendix 1.

Figure 3.1. Molecular maps of the binary vectors used for *A. thaliana* transformation. a. pSBS4140; b. pSBS4141; c. pSBS4142; d. pSBS4143; e. pSBS4144; f. pSBS4145; g. pSBS4146; h. pSBS4147 and i. pSBS4004 (Null Vector). Components of the T-DNA constructs: ACBP –*B. napus* cytosolic ACBP cDNA; At oleo gen- *A. thaliana* 18kDa oleosin gene; B82 – DNA fragment encoding 82 N'-terminal resi dues from BBE; OleoH3P – *A. thaliana* oleosin modified gene with short hydrophobic domain; D9 scFv- cDNA encoding single chain fragment of D9 antibody against *A. thaliana* 18kDa oleosin; PRS – DNA sequence encoding a signal peptide; KDEL – DNA fragment encoding KDEL retention signal; phaP and phaT – phaseolin promoter and terminator; ubiP and ubiT – ubiquitin promoter and terminator; pat – phosphinotricin acetyltransferase gene (selection marker responsible for the phosphinotricin tolerance of the transgenic plants); RB and LB – left and right borders of T-DNA.


a.
pSBS4140
12530 bp
RB
phaP
At oleo gen
Nco I (2499)
ACBP
Hin dIII (2779)
phaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
b.
pSBS4141
12567 bp
RB
phaP
Nco I (1740)
ACBP
Nco I (2016)
At oleo gen
Hin dIII (2816)
phaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
c.
pSBS4142
12767 bp
RB
Pst I (193)
PhaP
B82
Sph I (1919)
Sph I (2382)
At Oleo
ACBP
PhaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
d.
pSBS4143
12198 bp
RB
phaP
Oleo-H3P
Spe I (2153)
ACBP
Hin dIII (2443)
phaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
e.
pSBS4144
11858 bp
RB
phaP
PRS
Nco I (1815)
ACBP-KDEL
Hin dIII (2107)
phaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
f.
pSBS4145
12611 bp
RB
phaP
PRS
Scfv-D9
Xho I (2560)
Nco I (2568)
ACBP-KDEL
Hin dIII (2860)
phaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
g.
pSBS4146
11771 bp
RB
phaP
Nco I (1740)
ACBP
Hin dIII (2020)
phaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
h.
pSBS4147
12524 bp
RB
phaP
D9
Xho I (2485)
Nco I (2493)
ACBP
Hin dIII (2773)
phaT
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta
i.
pSBS4004
8724 bp
RB
ubiP
pat
ubiT
LB
SpecR
ColE1
rep
sta

Molecular cloning of the constructs designed for expression in *B. napus* developing seeds was performed by cloning ACBP-Oleo, OleoH3P-ACBP and ACBP expression cassettes including phaseolin promoter and terminator into the *EcoRI* site of the pGreen0029 plasmid. The resulting vectors were named pGreen0029-41, pGreen0029-43 and pGreen0029-46, respectively (Figure 3.2a-c).

In order to study the sub-cellular localization of the products of *BnACBP* genetic constructs, a number of GFP-fusion constructs were made for seed-specific expression in *A. thaliana.* Oleosin, ACBP-Oleosin, OleoH3P-ACBP, ACBP-KDEL and ACBP constructs were combined with DNA fragment encoding green fluorescent protein (GFP) for the N-terminal in-frame fusion. Details of the molecular cloning and vectors' maps are provided in the Appendix1.

All molecular constructs were sequenced before introduction of the binary vectors conferring corresponding ACBP expression cassettes into *Agrobacterium tumefaciens* EHA101 (*A. thaliana* transformation) or GV3101 (*B. napus* transformation) through electroporation. Standard protocols available for the transformation of *Agrobacterium* were used (Clough and Bent, 1998). pGreen0029-derived vectors were mixed with pSoup plasmid for stable *Agrobacterium* transformation. pSoup is required for *in trans* replication of pGreen-based vectors in *Agrobacterium* (Hellens *et al.*, 2000) (Figure 3.2d). Presence of the vector conferring T-DNA of interest in *Agrobacterium* was confirmed by PCR with vector specific primers 385 and 1109 (Table A1.1).

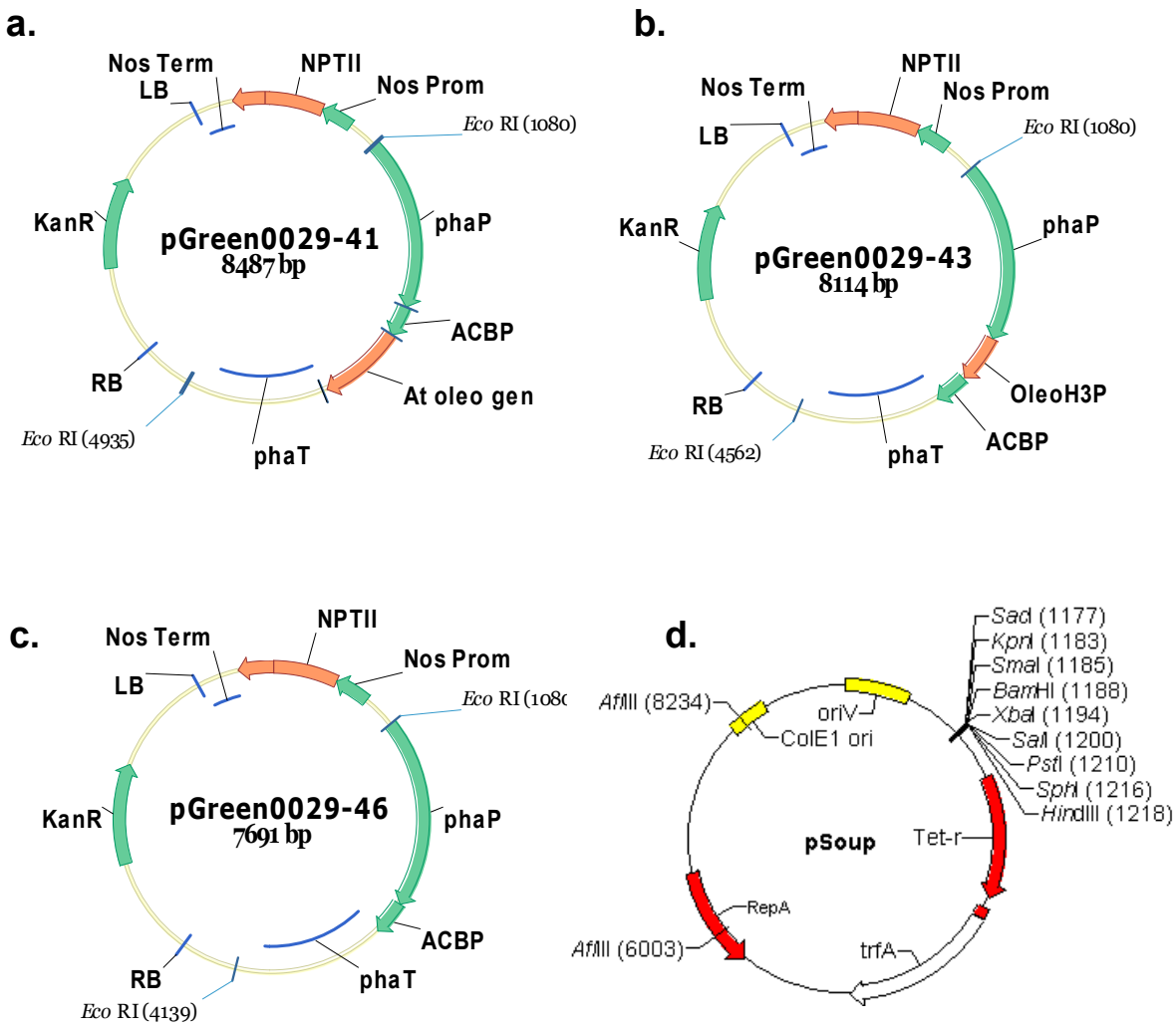


Figure 3.2. Molecular maps of the binary vectors used for *B. napus* transformation a. pGreen0029-41; b. pGreen0029-43; c. pGreen0029-46 and d. pSoup. Components of the T-DNA constructs: ACBP –*B. napus* cytosolic ACBP cDNA; At oleo gen- *A. thaliana* 18kDa oleosin gene; OleoH3P – *A. thaliana* oleosin modified gene with short hydrophobic domain; phaP and phaT – phaseolin promoter and terminator; Nos Prom and Nos Term– nopaline synthase promoter and terminator; NPTII – neomycin phosphotransferase II gene (selection marker responsible for the kanamycin tolerance of the transgenic plants); RB and LB – left and right borders of T-DNA.

3.2.2 Plant transformation and growth conditions

A. thaliana plants (C-24) were grown in Sunshine LA4 soil mix (SunGro, Vancouver, BC, Canada) in a growth chamber with a 16 hour light period at 350 μE light intensity and constant temperature of 20°C. *Agrobacterium tumefaciens* – mediated transformation of *A. thaliana* was performed using a floral dip method (Clough and Bent, 1998). The dipped plants were placed under a dome for 16 to 24 hours to maintain high humidity, and grown normally until seeds became mature. T_1 seeds were germinated and transformed seedlings were identified on the selective medium containing DL-phosphinothricin or Kanamycin. The herbicide resistant T_1 plants were transferred to soil seven days after germination and grown individually in growth chamber to produce mature T_2 seeds for seed oil analysis. T_2 seeds represent a mixture of heterozygous and homozygous genotypes for the presence of the transgene, while some (not more than 25%) are "Null-Segregates" – seeds with WT genotype. Four lines of the T_2 seeds per construct were selected and germinated on the selection medium. 10 T_2 plants per line were transferred to soil and produced T_3 seeds, which were harvested and used for analysis of seed oil and acyl-CoA pool FA composition and levels of the recombinant protein. T_3 seeds were also used for germination test on the selection medium to determin the zygosity of the line (Figure 3.3).

Constructs pGreen-41, pGreen-43 and pGreen-46 were used to transform *B. napus* L. cv DH12075. Transformation of *B. napus* was performed at Alberta Research Council (Vegrville, AB) following the method of Moloney *et al.* (1989).

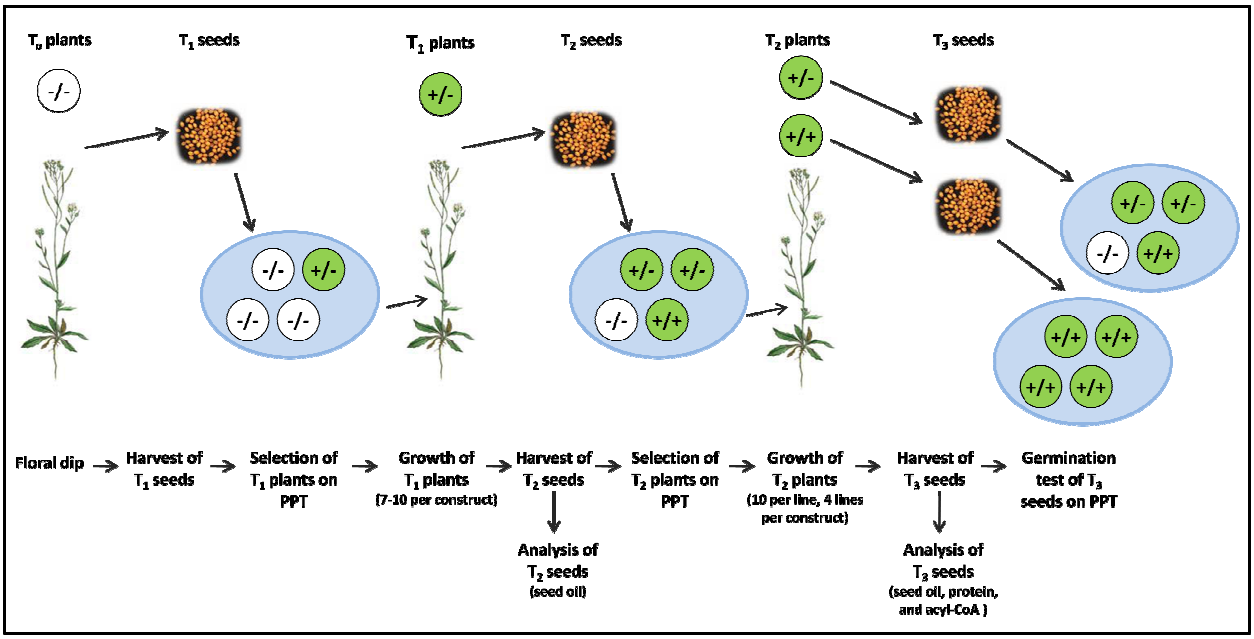


Figure 3.3. Schematic drawing of the production and selection of *A. thaliana* T_2 and T_3 seeds. T_1 seeds were harvested from T_0 plants that were subjected to a floral dip. Seven to ten T_1 plants per construct were identified on the selection medium with PPT and transplanted to soil. T_2 seeds were harvested separately for each plant and used for seed oil analysis (7-10 lines/insertion events per construct). Four lines with the highest PUFA content were selected for each construct and T_2 seeds corresponding to these lines germinated on selection medium with phosphinotricin (PPT) and ten T_2 plants per line (4 lines per construct) transplanted to soil. T_3 seeds were harvested for each plant separately and used for analysis of seed oil, acyl-CoA composition and the recombinant protein content. Sixty T_3 seeds for each plant were used for germination test to identify the zygosity of the parent T_2 plant. (+ and – signs represent the zygosity for the transgene).

3.2.3 Biochemical analysis of seed oil

Seed oil was extracted from 10 mg of mature T_2 seeds using a hexane – isopropanol method (Hara and Radin, 1978). Seed oil content was determined by gravimetric method with four technical replicates per T_2 line. Methanolic HCl was used as a methylation agent for preparing FA methyl esters (FAMEs) for subsequent separation by gas chromatography (GC). Internal standards (tripentadecanoylglycerol, 15:0-TAG; triheptadecanoylglycerol, 17:0-TAG) were added to each sample before seed oil extraction and before FA methylation, respectively, to account for sample loss in these procedures, and to estimate the total FA content by GC. Direct transmethylation of ground seeds with methanolic HCl was used for seed oil analysis of T_3 seeds. FAMEs were analyzed on Agilent 6890N Gas Chromatograph with 5975 inert XL Mass Selective Detector equipped with an autosampler (Agilent, Wilmington, DE, USA). FAMEs were separated using a capillary DB-23 (30m) column (0.25 mm x 0.25 µm x 30 m) with constant He flow 1.2 mL/min and a temperature program: 90-180°C at 10°C/min, hold at 180 for 5 min, 180-230°C at 5°C/min. Integration events were detected and identified between 9 and 20 min and compared against a NuChek 463 gas–liquid chromatography standard (NuChek, Elysian, MN, USA).

3.2.4 Statistical analysis

SAS 9.1 software (SAS Institute Inc., Cary, NC, USA) was used for performing all statistical procedures. FA profile, defined here as a composition of the FA classes (saturated, monounsaturated and polyunsaturated), and FA composition (16:0, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 22:1) of seed oil or acyl-

CoA pool of each construct were compared to WT using ANOVA analysis (GLM procedure) with Dunnett's test. Four biological replicates (different insertion events) per construct were used in the analysis.

3.2.5 Analysis of the BnACBP levels in *A. thaliana* developing and mature seeds

Developing embryos from 6 siliques collected at 16 days after flowering (DAF) or 40 mature *Arabidopsis* seeds (25 mg) were ground in 0.5 mL of extraction buffer (0.4 M sucrose, 0.5 M NaCl, and 50 mM Tris-HCl, pH 8.0) and the total seed proteins (TSP) were solubilized with addition of 10% SDS (to a final concentration of 2% SDS) and boiled for 10 minutes. Total protein content was determined using BCA protein assay (Pierce, Rockford, IL, USA). TSP were then analyzed by SDS-PAGE using standard protocols (Sambrook et al., 1989) and stained with Coomassie Brilliant Blue R 250 or blotted for Western analysis. Blotted samples were probed with polyclonal antibody directed against *Brassica napus* ACBP (Brown et al., 1998) or monoclonal antibody directed against *Arabidopsis thaliana* 18 kDa oleosin (raised by SemBioSys Genetics Inc., Calgary, AB, Canada). ACBP was detected using secondary donkey anti-rabbit IRDye® 800 CW (LiCor Biosciences, Lincoln, NE, USA) and analyzed using the Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE, USA). The 18 kDa oleosin monoclonal antibody was used in three capacities, one to detect transgene products where the ACBP is fused to the ACBP, secondly as an internal standard to normalize for the equal sample loading, and finally as an internal standard to determine transgene expression levels because the endogenous 18 kDa

oleosin expressed at a level equivalent to 1.5% of the total seed protein in mature *Arabidopsis* seeds (Nykiforuk et al., 2006). The 18 kDa oleosin or oleosin fusion products was detected using secondary goat anti-mouse IRDye® 800 CW (LiCor Biosciences, Lincoln, NE) and analyzed using the Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE).

3.2.6 Analysis of the acyl-CoA pool of *A. thaliana* developing seeds

Derivatization and analysis of the acyl-CoA pool of *A. thaliana* developing seeds was done according to the technique described by Larson and Graham (2001). Developing seeds (10, 15 or 20 DAF) from 5-6 frozen siliques were placed in a centrifuge tube with a 3mm glass bead (Fisher Scientific) and homogenized on a bead beater (Biospec Products Inc.) for 1 minute. 200 µL of freshly prepared extraction buffer (2 ml iso-propanol, 2 mL 50 mM KH_2PO_4 pH 7.2, 50 µL glacial acetic acid, 80 µL 50 mg/ml essentially FA free BSA) and 400 µl of petroleum ether saturated with 1:1 isopropanol:water were added to each tube. Samples were vortexed and spun, and the upper phase was discarded. The lower phase was washed with 400 µL of petroleum ether saturated with 1:1 isopropanol:water another 3-4 times. Finally, 5 µL of saturated ammonium sulphate and 600 µL of 2:1 (V/V) methanol:chloroform was added to the washed lower phase. Samples were mixed, incubated at room temperature for 20 min and centrifuged at 12,000 x g for 2 min. The supernatant was transferred to 2 mL HPLC vials and dried under vacuum (Automatic Environment SpeedVac with VaporNet AES200, Savant). When dry, 100 µL of the derivatizing agent (0.5M chloroacetaldehyde in 0.15M trisodium citrate/citric acid pH 4.0, 0.5% SDS) were

added to each vial and incubated for 20 min at 85°C. Afterwards the reaction mixture was filtered through a 0.45 μm nylon filter in a 2 mL tube and transferred back to the HPLC vial with a spring insert. Samples were analyzed on an Agilent 1200 HLPC (Agilent Technologies, Wilmington, DE, USA) with LUNA-18(2) (150mm x 2mm x 5μ) column, using the method described in Larson and Graham (2001).

3.2.7 Confocal microscopy

Morphological analysis of the sub-cellular localization of GFP-fusion constructs in *A. thaliana* embryo cells was performed *in vivo*, using dark field confocal microscopy. *A. thaliana* developing embryos (16 DAF) were isolated by incubating seeds in PBS solution (Phosphate buffer 100 mM, pH 7.2, NaCl 0.7% w/v) and pressing them gently with a glass microscope slide cover. Embryos were transferred to the microscope slide by pipette and covered with a slide cover. Embryos were examined with Leica TCS-SP2 Multiphoton Confocal Laser Scanning Microscope (TCS-MP) with 488 nm laser excitation and 510 nm emission.

3.3 Results

3.3.1 Molecular cloning of the *BnACBP* constructs and plant transformation

The molecular cloning of eight genetic constructs for seed-specific expression of *B. napus* cDNA encoding a 10 kDa ACBP in *A. thaliana* was performed for seed-specific protein expression (Figure 3.4). In order to examine

the effect of BnACBP on seed oil formation it was logical to express this protein in developing seeds during active TAG accumulation and at the cellular location where seed oil biosynthesis occurs.

Four out of eight genetic constructs were designed to be targeted to the surface of the ER, where the reactions of the Kennedy pathway are located. Expression of transgenic protein on the ER was mediated by means of the gene fusion to the *A. thaliana* 18 kDa oleosin gene, native or modified. In constructs Oleo-ACBP and ACBP-Oleo, BnACBP cDNA was cloned in frame with the *A. thaliana* 18 kDa oleosin gene for N'- and C'-terminal fusion, respectively (Figure 3.4, constructs 1 and 2).

Since oleosin synthesis is targeted to the ER membrane, the fusion protein is expected to be targeted to this cellular location also, with subsequent translocation to the oil body (OB). For the purposes of this project, however, it was desirable to keep acyl-CoA binding sites on the surface of the ER for the duration of TAG biosynthesis during seed development. Two modified oleosin genes were used to disrupt/ decrease oleosin-ACBP fusion targeting to OB. The first modification (OleoH3P) involved removing of 32 amino acids from the hydrophobic domain of oleosin, which was to promote a more stable conformation of the hydrophobic stretch within the lipid bilayer of the ER membrane (Construct OleoH3P-ACBP) (Figure 3.4, construct 4). Another oleosin modification involved addition of the luminal domain from *Papaver somniferum* berberine bridge enzyme (BBE) at the N'-terminus (B82-Oleosin-ACBP) (Figure 3.4, construct 3). These extra 82 amino acid residues form a transmembrane domain were expected to act as an "anchor"

facilitating retention of the Oleosin-ACBP fusion protein on the ER membrane. It has been shown that oleosin membrane-straddled topologies with N-terminal sequence in the ER lumen and C-terminal sequence in the cytosol were unable to target to oil bodies efficiently, instead these variants accumulated proportionately higher in ER microsomal fractions, demonstrating a block in transferring from ER to oil bodies (Abell *et al.*, 2004).

Cytosolic BnACBP was also produced at its native cellular location (Construct ACBP) (Figure 3.4, construct 7). One more cytosolic construct was made by ligating *BnACBP* with cDNA encoding for a single chain variable fragment of the antibody against *A. thaliana* 18 kDa oleosin (Construct D9-ACBP) (Figure 3.4, construct 8). This last fusion construct was expected to function as a shuttle that acquires acyl-CoA in the cytosol and delivers it to the surface of ER and OB due to D9 affinity towards oleosin.

Two genetic constructs with N-terminal signal peptide and C-terminal ER retention signal (KDEL) were made in order to target ACBP and D9-ACBP fusion protein to the ER lumen (Constructs ACBP-KDEL and D9-ACBP-KDEL, Figure 3.4) (Okamoto *et al.*, 2003). Because of the luminal residence, the transgenic polypeptides resulting from these two constructs were not to be in direct contact with the cytosolic acyl-CoA pool, and thus no effects on seed oil formation were expected.

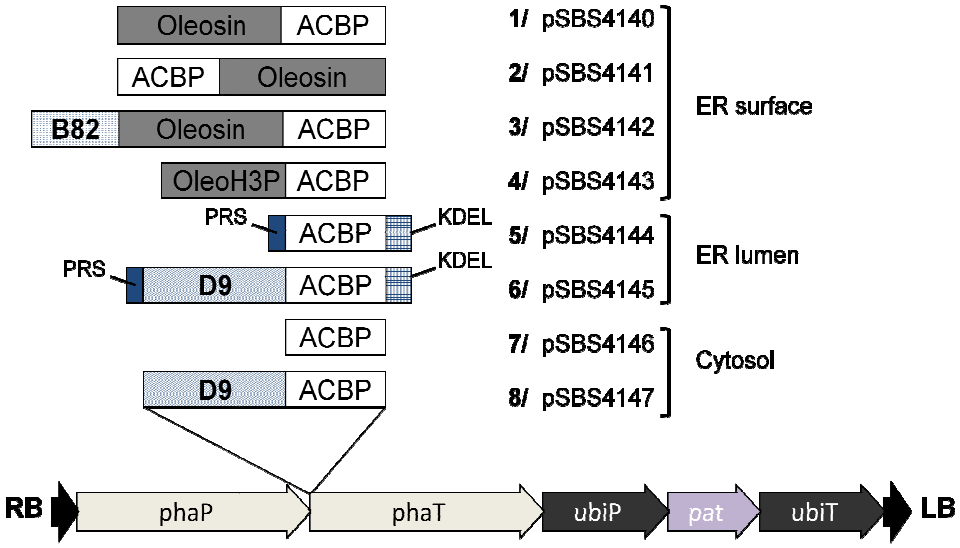


Figure 3.4. *BnACBP* molecular constructs targeted to different cellular locations. ACBP - *B. napus* cytosolic ACBP cDNA; Oleosin - *A. thaliana* 18kDa oleosin gene; OleoH3P – cDNA encoding oleosin variant with short hydrophobic domain; D9 – cDNA encoding a single chain variable fragment of the antibody against 18kDa oleosin; B82 – cDNA encoding N'-terminal lumenal domain from BBE; KDEl – DNA encoding KDEL retention signal; PRS – targeting sequence for ER expression. All constructs were cloned into phaseolin promoter: terminator expression cassette (phaP-phaT). T-DNA contained a phosphinotricin acetyltransferase gene expression cassette under control of ubiquitin promoter and terminator (ubiP-pat-ubiT) as a selection marker. Names of the binary vectors and expected cellular localization of expression corresponding to each constructs are given on the right.

3.3.2 Seed-specific expression of the cDNA encoding 10 kDa cytosolic BnACBP affects fatty acid composition of seed oil in *A. thaliana*

Analysis of *A. thaliana* T_2 seed oil was done in 4 technical replicates per T_2 line represented by the seeds from the single T_1 plant. Each T_1 plant represented a unique insertion event. A varying number of lines (7-10) were analyzed individually for each construct, depending on the number of T_1 plants regenerated and the amount of T_2 seeds harvested per plant. Four T_2 lines with similar phenotype (similar levels of variation of FA composition of seed oil) were used for statistical data analysis. Construct Oleo-ACBP produced only one transgenic line and was excluded from the analysis due to the lack of replicates.

Analysis of seed oil from T_2 lines revealed a significant increase in $18{:}2^{cis\Delta 9,12}$ (18:2) and a decrease in $20{:}1^{cis\Delta 11}$ (20:1) and 18:0 in all lines transformed with *BnACBP* (Table 3.1). All constructs also had decreased levels of $18{:}3^{cis\Delta 9,12,15}$ (α-18:3) in seed oil compared to WT, except for ACBP construct, where α-18:3 content went up. Some non-specific changes in FA composition of seed oil were also observed, and these were accounted for with the help of the lines transformed with Null Vector. It was previously observed that *Agrobacterium*-mediated transformation of *A. thaliana* with genes not directly involved in lipid metabolism could cause significant changes in seed oil composition, particularly in 18:1 and α-18:3 levels (Jim Metz, Market BioSciences, Boulder, CO, USA, personal communication).

Table 3.1. FA composition of the seed oil from *A. thaliana* T_2 seeds transformed with *BnACBP* constructs. Four T_2 lines with the highest PUFA% in seed oil within each construct were included in the analysis. Mean %weight ± SD of the means (n=4). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}/\lozenge$) values significantly greater/ smaller than WT at α=0.1.

Construct	16:0	18:0	18:1	18:2	18:3	20:1
WT	7.38 ± 0.05	3.66 ± 0.04	15.51 ± 0.06	26.79 ± 0.08	17.04 ± 0.14	21.06 ± 0.04
Null Vector	7.73 ± 0.19	3.30 ± 0.71	16.78 ± 0.27	26.84 ± 2.13	15.87 ± 0.70	21.18 ± 0.79
ACBP-Oleo	8.11 ± 0.39$^{\Delta}$	2.46 ± 0.19$^{\blacktriangledown}$	15.27 ± 1.05	32.40 ± 0.33$^{\blacktriangle}$	15.68 ± 0.89◊	17.90 ± 0.85$^{\blacktriangledown}$
OleoH3P-ACBP	7.79 ± 0.25	2.52 ± 0.14$^{\blacktriangledown}$	16.65 ±1.24	31.43 ± 0.21$^{\blacktriangle}$	15.72 ± 1.06◊	18.18 ± 0.29$^{\blacktriangledown}$
B82-Oleo-ACBP	9.53 ± 0.90$^{\blacktriangle}$	2.22 ± 0.26$^{\blacktriangledown}$	15.04 ± 1.02	35.43 ± 2.00$^{\blacktriangle}$	15.10 ± 1.31$^{\blacktriangledown}$	16.32 ± 1.95$^{\blacktriangledown}$
ACBP-KDEL	7.96 ± 0.34	2.64 ± 0.18$^{\blacktriangledown}$	16.04 ± 0.84	33.40 ± 0.72$^{\blacktriangle}$	12.90 ± 0.19$^{\blacktriangledown}$	19.67 ± 0.56◊
D9-ACBP-KDEL	8.26 ± 0.55$^{\blacktriangle}$	2.47 ± 0.09$^{\blacktriangledown}$	18.21 ± 0.97$^{\blacktriangle}$	32.11 ± 0.58$^{\blacktriangle}$	12.15 ± 0.27$^{\blacktriangledown}$	19.03 ± 0.81$^{\blacktriangledown}$
ACBP	6.64 ± 0.35◊	2.57 ± 0.25$^{\blacktriangledown}$	16.28 ± 0.36	29.78 ± 0.72$^{\blacktriangle}$	19.04 ± 1.32$^{\blacktriangle}$	17.71 ± 0.55$^{\blacktriangledown}$
D9-ACBP	7.63 ± 0.10	2.41 ± 0.11$^{\blacktriangledown}$	16.85 ± 1.07	31.00 ± 0.55$^{\blacktriangle}$	14.45 ± 0.52$^{\blacktriangledown}$	19.91 ± 0.33◊

Constructs containing the oleosin gene (ACBP-Oleo, OleoH3P-ACBP and B82-Oleo-ACBP) affected FA composition in a similar way, resulting in an increase in 18:2 (Table 1), decrease in 20:1 (to 16.32% from 21.06 in WT) and 18:0 (to 2.22% from 3.66% in WT). α-18:3 was decreased only slightly in seeds transformed with these constructs, and was very similar to Null Vector levels. Plants harbouring ACBP-Oleo and B82-Oleo-ACBP also displayed some increase in 16:0 in seed oil.

The next group that had some similarities in resulting changes of FA composition were constructs with the KDEL retention signal (ACBP-KDEL and D9-ACBP-KDEL). Like the other constructs, KDEL constructs resulted in an increase of 18:2 and a decrease of 18:0 in seed oil, but also had some rather unique effects, such as a decrease in α-18:3 beyond what was observed in null vector seeds. Also, the decrease in 20:1 was more subtle compared to constructs with oleosin.

D9-ACBP had the least effect on FA composition of seed oil, which might be due to the low stability of the polypeptide. It has been documented that single-chain antibodies are not very stable when expressed as soluble polypeptides in the cytoplasm (Eto *et al*., 2003). Low levels of the D9-ACBP protein were also confirmed by the Western analysis, which will be discussed in detail later. The increase in 18:2 and decrease in 20:1 were of the least magnitude compared to the rest of the constructs.

In the ACBP construct, α-18:3 increased up to 19.04% compared to 17.04% in WT or 15.87% in the Null Vector. Another effect specific to the ACBP

construct was a decrease in 16:0, which resulted in an overall decrease of SFA content from 13.4% in WT to 10.85% in ACBP transgenic seeds.

Overall, T_2 lines transformed with constructs ACBP-Oleo, OleoH3P-ACBP, ACBP-KDEL, ACBP and B82-Oleo-ACBP showed a significant increase in PUFA compared to WT (Table 3.2). The increase in PUFA content in seed oil appeared to happen at the expense of MUFA, and to some extent SFA in OleoH3P-ACBP, ACBP-KDEL and ACBP. Four lines with the highest PUFA content were selected for each construct and used for producing the next generation of seeds. Four T_3 lines that originated from the transgenic parent (ACBP construct) and segregated back to the wild-type genotype were also selected and included in the analysis as a null-segregate control.

Analysis of T_3 seeds expressing *BnACBP* constructs confirmed our previous findings of this heritable trait. T_3 seeds obtained from T_2 lines transformed with construct ACBP-Oleosin, OleosinH3P-ACBP, B82-Oleo-ACBP, ACBP-KDEL and ACBP showed significant increases in PUFA compared to WT (Table 3.3). Just as in T_2 seeds, PUFA content elevation in T_3 seeds was due to an increase in 18:2 and decrease in 20:1 and 18:0 (Table 3.4). A decrease in α-18:3 and an increase in 16:0 were also observed in most constructs. T_3 seeds transformed with ACBP construct once again showed some unique changes, such as a decrease in 16:0 and an increase is α-18:3. Statistical analysis performed on mol% values gave the same results as analysis with %weight (Appendix 2).

Table 3.2. Composition of FA classes in *A. thaliana* T_2 seeds transformed with *BnACBP* constructs. Four lines with the highest PUFA% in seed oil within each construct are included in the analysis Mean %weight ± SD of the means (n=4). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05

Construct	SFA	MUFA	PUFA
WT	13.40 ± 0.11	41.23 ± 0.09	45.36 ± 0.20
Null Vector	13.29 ± 1.41	42.61 ± 0.75	44.10 ± 1.60
ACBP-Oleo	12.54 ± 0.45	37.85 ± 1.18$^{\blacktriangledown}$	49.60 ± 0.86$^{\blacktriangle}$
OleoH3P-ACBP	11.96 ± 0.50$^{\blacktriangledown}$	39.57 ± 1.46$^{\blacktriangledown}$	48.46 ± 0.97$^{\blacktriangle}$
B82-Oleo-ACBP	13.10 ± 0.96	35.37 ± 1.95$^{\blacktriangledown}$	51.52 ± 1.22$^{\blacktriangle}$
ACBP-KDEL	12.15 ± 0.51$^{\blacktriangledown}$	40.21 ± 1.18	47.63 ± 0.81$^{\blacktriangle}$
D9-ACBP-KDEL	12.23 ± 0.73	42.18 ± 1.34	45.57 ± 0.73
ACBP	10.85 ± 0.69$^{\blacktriangledown}$	38.77 ± 0.52$^{\blacktriangledown}$	50.37 ± 0.69$^{\blacktriangle}$
D9-ACBP	11.62 ± 0.16$^{\blacktriangledown}$	41.54 ± 0.49	46.83 ± 0.36

Table 3.3. Composition of FA classes in *A. thaliana* T_3 seeds transformed with *BnACBP* constructs. Mean %weight ± SD of the means (n=4). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}/\Diamond$) values significantly greater/ smaller than WT at α=0.1.

Construct	SFA	MUFA	PUFA
WT	13.40 ± 0.11	41.23 ± 0.09	45.36 ± 0.20
Null Segregate	12.78 ± 0.11	41.10 ± 0.44	46.11 ± 0.34
ACBP-Oleo	12.56 ± 0.39$^{\blacktriangledown}$	38.84 ± 1.41$^{\blacktriangledown}$	48.60 ± 1.22$^{\blacktriangle}$
OleoH3P-ACBP	12.63 ± 0.30$^{\blacktriangledown}$	38.94 ± 1.27◊	48.42 ± 0.97$^{\blacktriangle}$
B82-Oleo-ACBP	13.38 ± 0.46	40.34 ± 1.09◊	46.27 ± 0.66$^{\Delta}$
ACBP-KDEL	12.97 ± 0.25	41.04 ± 1.26	45.98 ± 1.01
D9-ACBP-KDEL	12.23 ± 0.47$^{\blacktriangledown}$	43.16 ± 2.12	44.60 ± 1.66
ACBP	11.12 ± 0.12$^{\blacktriangledown}$	40.06 ± 0.99◊	48.81 ± 0.78$^{\blacktriangle}$
D9-ACBP	11.49 ± 0.21$^{\blacktriangledown}$	43.25 ± 0.78	45.24 ± 0.60

Table 3.4. FA composition of seed oil of *A. thaliana* T_3 seeds transformed with *BnACBP* constructs. Mean %weight ± SD of the means (n=4). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}/\Diamond$) values significantly greater/ smaller than WT at α=0.1

Construct	16:0	18:0	18:1	18:2	18:3	20:1
WT	7.38 ± 0.06	3.67 ± 0.04	15.51 ± 0.06	26.79 ± 0.08	17.03 ± 0.14	21.06 ± 0.04
Null Segregate	7.51 ± 0.04	3.25 ± 0.08	15.69 ± 0.71	27.62 ± 0.31	16.92 ± 0.57	20.67 ± 0.31
ACBP-Oleo	8.15 ± 0.32$^{\blacktriangle}$	2.44 ± 0.08$^{\blacktriangledown}$	16.01 ± 1.50	31.53 ± 1.90$^{\blacktriangle}$	15.68 ± 1.57$\Diamond$	17.98 ± 1.01$^{\blacktriangledown}$
OleoH3P-ACBP	8.28 ± 0.28$^{\blacktriangle}$	2.61 ± 0.02$^{\blacktriangledown}$	16.71 ± 0.97	31.51 ± 0.41$^{\blacktriangle}$	15.63 ± 0.65$\Diamond$	17.62 ± 0.40$^{\blacktriangledown}$
B82-Oleo-ACBP	7.88 ± 0.36$^{\blacktriangle}$	3.05 ± 0.01$^{\blacktriangledown}$	16.48 ± 0.47	29.61 ± 1.20$^{\blacktriangle}$	15.14 ± 0.58$\Diamond$	18.54 ± 1.43$^{\blacktriangledown}$
ACBP-KDEL	8.62 ± 0.25$^{\blacktriangle}$	2.72 ± 0.13$^{\blacktriangledown}$	17.13 ± 1.11	32.66 ± 1.52$^{\blacktriangle}$	12.14 ± 1.11$^{\blacktriangledown}$	19.36 ± 0.97$^{\blacktriangledown}$
D9-ACBP-KDEL	8.05 ± 0.30$^{\blacktriangle}$	2.49 ± 0.09$^{\blacktriangledown}$	18.87 ± 2.40$^{\blacktriangle}$	30.47 ± 0.29$^{\blacktriangle}$	12.95 ± 1.70$^{\blacktriangledown}$	19.69 ± 0.30$\Diamond$
ACBP	6.93 ± 0.11$\Diamond$	2.59 ± 0.21$^{\blacktriangledown}$	16.86 ± 0.65	28.89 ± 0.73$^{\blacktriangle}$	18.36 ± 0.73$^{\Delta}$	18.18 ± 0.75$^{\blacktriangledown}$
D9-ACBP	7.47 ± 0.08	2.49 ± 0.13$^{\blacktriangledown}$	18.64 ± 0.95$^{\blacktriangle}$	30.21 ± 0.71$^{\blacktriangle}$	13.77 ± 1.16$^{\blacktriangledown}$	20.32 ± 0.36

The magnitude of the FA composition changes, however, was more subtle in the T_3 seeds compared to the changes observed in the T_2 data. Results obtained from both data sets point out the major effect of the *ACBP* transgene on the FA composition of seed oil, which was an increase in the proportions of 18:2 (and α-18:3 for constract ACBP) and a decrease in the proportion of 20:1.

Since this effect was observed in all plants expressing ACBP constructs, but to a different degree compared to WT, it was important to find out if the extent of the transgene effect on FA composition was dependent on the levels of transgenic ACBP in mature and developing seeds.

3.3.3 Recombinant ACBP levels in *A. thaliana* T_3 mature and developing seeds

Levels of the recombinant ACBP were studied in mature and developing (16 DAF) T_3 seeds using Western blot analysis. Approximately 2 μg of the total seed protein (TSP) per sample were loaded on SDS-PAGE. Each sample was run on two different gels at the same time. One gel was used for the Western with BnACBP specific antibodies (Figures 3.5a) and the other gel for analysis with D9 antibodies against 18 kDa oleosin (Figures 3.5b). BnACBP specific antibodies did not cross-react with native ACBP in *Arabidopsis*. Oleosin was used as an internal standard to normalize for equal sample loading against the amount of the control sample (WT).

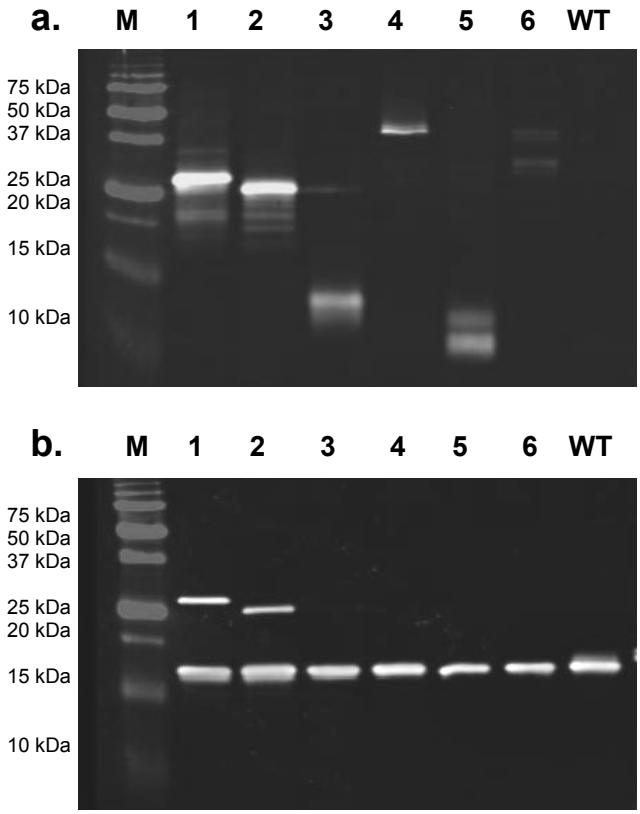


Figure 3.5. Western analysis of the total seed protein (TSP) from *A. thaliana* T_3 seeds transformed with *BnACBP* constructs. a. Blot incubated with polyclonal antibodies against BnACBP. b. Blot incubated with monoclonal antibodies against 18kDa AthOleosin. M – Protein marker (Precision Plus Protein™ Standards, Bio-Rad); 1 – ACBP-Oleo (28 kDa); 2 – OleoH3P-ACBP (25 kDa); 3 – ACBP-KDEL (13.7 kDa); 4 – D9-ACBP-KDEL (40.7 kDa); 5 – ACBP (10 kDa); 6 – D9-ACBP (37 kDa); WT – wild type.

Analysis of the mature T_3 seeds (10 lines per construct analyzed, 4 lines per construct included in statistical data analysis) confirmed that all ACBP constructs were expressed and were present at detectable levels (Figure 3.6). The highest concentration of the transgene was observed for the constructs that contained oleosin as a fusion partner (0.819 ± 0.162 and 0.626 ± 0.066 μmol/g TSP for constructs ACBP-Oleosin and OleosinH3P-ACBP, respectively). Constructs containing KDEL retention signal were found in mature seed at much lower levels (0.185 ± 0.071 and 0.112 ± 0.051 μmol/g TSP for constructs ACBP-KDEL and D9-ACBP-KDEL respectively), while the cytosol-targeted constructs had even lower concentrations in mature seeds (0.027 ± 0.006 and 0.002 ± 0.001 μmol/g TSP for constructs ACBP and D9-ACBP, respectively). The levels of ACBP transgene product were also analysed in 16 DAF developing seeds corresponding to the peak of expression from the phaseolin promoter in *A. thaliana* seeds. Surprisingly, we found that ACBP levels in developing seeds were not significantly different from those observed in mature seeds (Figure 3.6).

We observed a strong positive correlation between the recombinant ACBP levels in developing seeds and seed oil PUFA content for all analyzed constructs except for ACBP construct (Figure 3.7). No clear correlation, however, was detected between 18:2 content in seed oil and BnACBP levels, which suggests that factors in addition to the absolute amount of transgenic protein in the seeds may have a strong effect on the FA composition of seed oil. One of those factors could be subcellular localization of the transgenic protein.

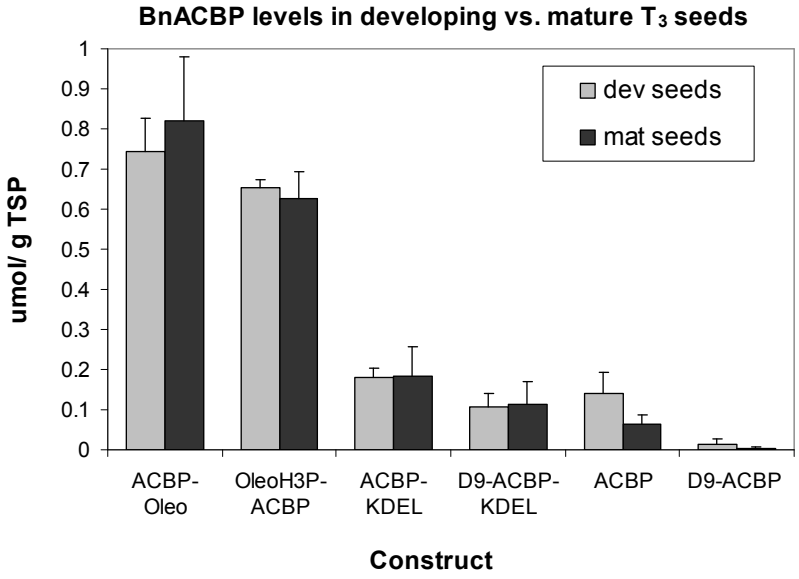


Figure 3.6. Levels of BnACBP in *A. thaliana* developing (16 DAF) and mature T_3 seeds transformed with *BnACBP* constructs. Mean ± SD (n=4).

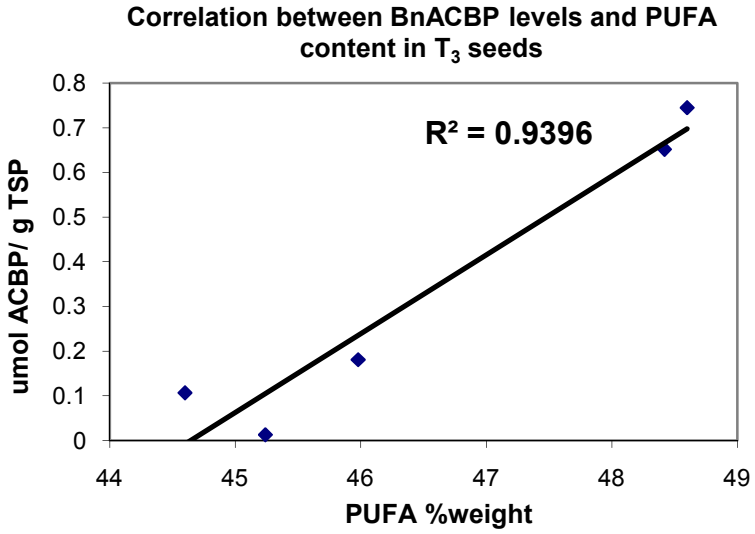


Figure 3.7. Positive correlation between proportion of PUFA in seed oil in mature seeds and levels of BnACBP in *A. thaliana* developing (16 DAF) T_3 seeds transformed with *ACBP-Oleo, OleoH3P-ACBP, ACBP-KDEL, D9-ACBP* and *D9-ACBP-KDEL*.

3.3.4 Subcellular localization of recombinant protein-GFP fusions

GFP cDNA was fused in frame with constructs ACBP-Oleosin, OleosinH3P-ACBP, ACBP-KDEL and ACBP at the N'-terminus. These four constructs were designed as a representation of ACBP fusion with native oleosin, modified oleosin, KDEL, and ACBP by itself. GFP-Oleosin and GFP constructs were used as controls.

Confocal microscopy imaging of the developing embryos transformed with GFP constructs appeared to indicate that ACBP fusions with native and modified oleosin (H3P modification) are targeted to oil bodies (Figure 3.8a-c). GFP-ACBP-DKEL fusion protein appeared on the membrane structures within the cell, which most likely represents endoplasmic reticulum (Figure 3.8d). With the microscopic technique used in this analysis it was not possible to distinguish between the luminal and cytosolic sides of the membrane structures. GFP-ACBP fusion as expected was observed in both cytosol and nucleus (Figure 3.8e). The nuclear localization of GFP-ACBP fusion is due to a small size of the complex, which enables it to migrate through nuclear pore complexes. GFP is efficiently transported into the nucleus (Figure 3.8f) and can transport small fusion proteins with it. The presence of GFP-ACBP in the nucleus, however, does not reflect the native localization of ACPB, which was recently demonstrated by Chen et al., 2008.

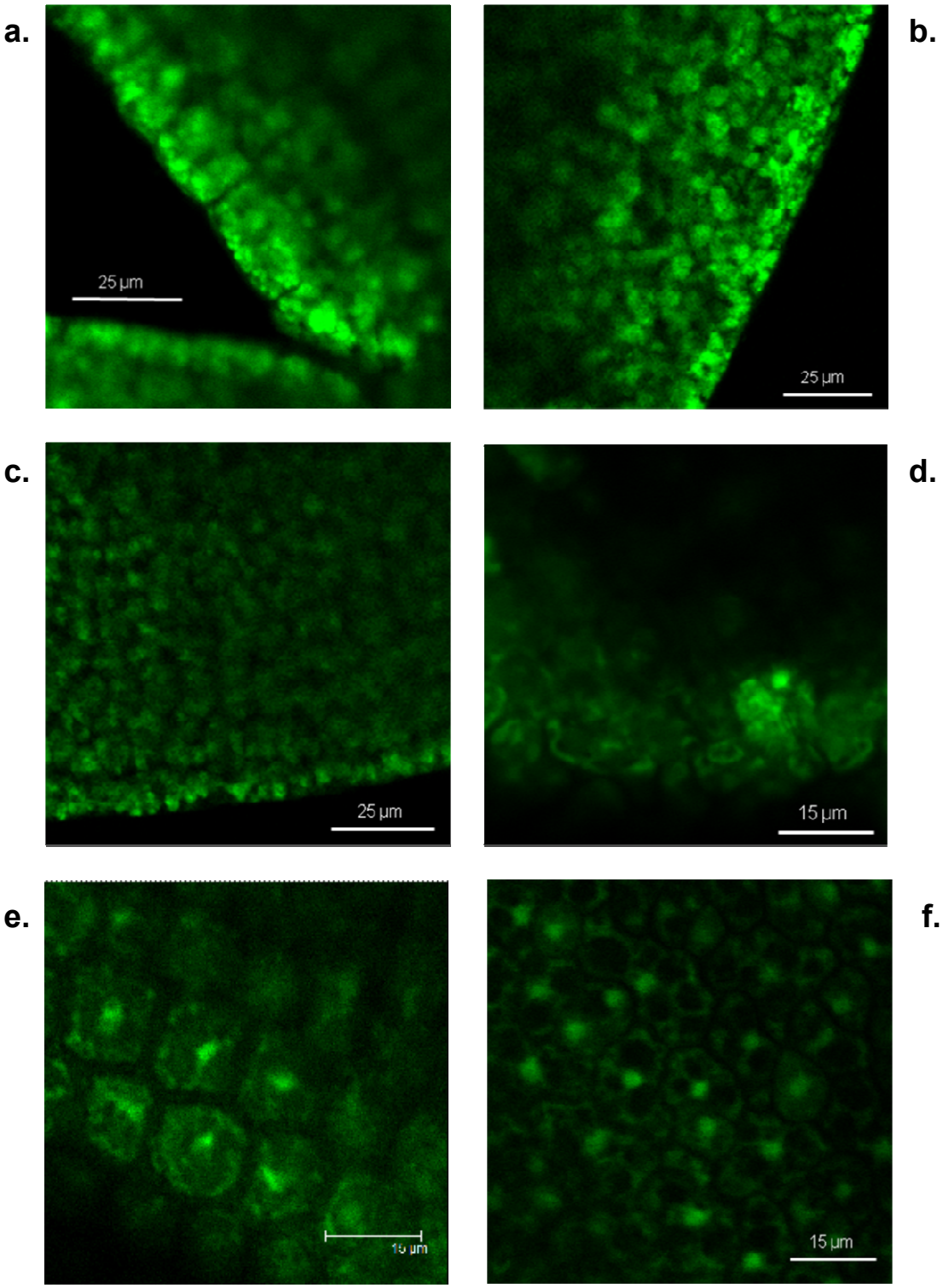


Figure 3.8. Subcellular localization of the GFP-fusion proteins in *A. thaliana* developing embryos. a. GFP-Oleo; b. GFP-ACBP-Oleo; c. GFP-OleoH3P-ACBP; d. GFP-ACBP-KDEL; e. GFP-ACBP; f. GFP.

3.3.5 Analysis of the acyl-CoA pool composition in *A. thaliana* T_3 developing seeds

Developing T_3 seeds were harvested at different developmental stages (10, 15 and 20 DAF) for 4 homozygous lines per construct. The acyl-CoA pool composition differed between these developmental stages both in WT and transgenic lines (Figure 3.9). Saturated acyl groups (C16:0, C18:0 and C20:0) represented the largest portion of the pool at 10 DAF, which began to decrease at later developmental stages. C20:0 levels decreased significantly from 33% to 6.7% in WT from 10 to 20 DAF. C18:0 also decreased, while C16:0 levels went up during the course of seed development. Levels of C18:1 were not significantly different in WT seeds over the tested developmental stages, but slightly decreased in transgenic plants. 20:1-CoA increased from day 10 to 15, but then decreased before 20 DAF for WT and most of the transgenic lines. A similar pattern was observed for 22:1. Levels of the polyunsaturated acyl groups (C18:2, C18:3 and C20:2) remained quite low at the early developmental stages (10-15 DAF). 18:2-CoA increased substantially, especially in transgenic seeds, from 15 to 20 DAF.

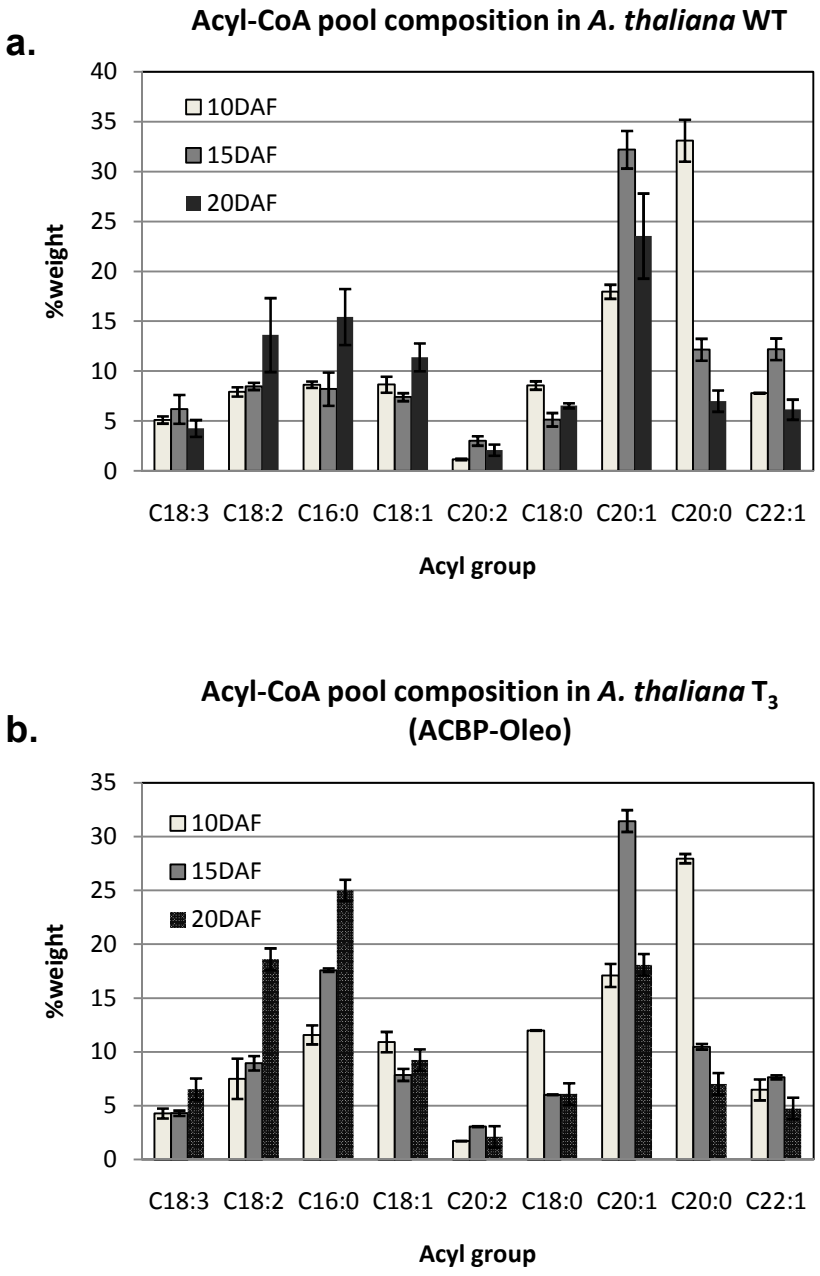


Figure 3.9. Composition of the total acyl-CoA pool from the developing *A. thaliana* seeds (10, 15 and 20 DAF). a. WT seeds. b. Transgenic seeds (expressing *ACBP-Oleo* construct).

When comparing the acyl-CoA pool composition of the transgenic seeds to the WT within each developmental stage, we observed some differences consistent with those in seed oil composition (Tables 3.5 – 3.7). At all three stages of development tested, transgenic seeds had considerably higher levels of C16:0 comparing to the WT. All transgenic lines, except those transformed with the ACBP construct, had decreased levels of C18:3 at 10 and 15 DAF compared to the WT (same pattern in seed oil). C18:2 levels in transgenic seeds were similar to those in WT at 10 and 15 DAF, but increased by 20 DAF in lines transformed with the soluble constructs (ACBP and D9-ACBP) and ACBP-KDEL construct (similar to effect in seed oil). The major acyl group at 10 DAF, C20:0, significantly decreased in transgenic seeds, while at later stages C20:1 and C22:1 were affected the most, having lower levels in transgenic lines compared to WT. Overall, the main changes in the acyl-CoA pool composition in transgenic lines were an increase in saturated groups (especially C16:0), an increase in 18:2 (especially in lines transformed with soluble ACBP constructs), and a decrease in very long chain acyl groups (C20:0, C20:1 and C22:1), which may suggest that higher levels of cytosolic ACBP affect the following processes: termination of acyl group synthesis/elongation and levels of polyunsaturated acyl groups in cytosol.

Table 3.5. Acyl composition of the total acyl-CoA pool from *A. thaliana* T_3 developing seeds (10 DAF) transformed with *BnACBP* constructs. Mean %weight ± SD (n=4). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}/\Diamond$) values significantly greater/ smaller than WT at α=0.1.

Construct	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:1
WT_10DAF	8.64 ± 0.31	8.56 ± 0.41	8.64 ± 0.81	7.91 ± 0.46	5.11 ± 0.36	33.10 ± 2.09	17.95 ± 0.71	7.8 ± 0.03
ACBP-Oleo	10.96 ± 1.61$^{\blacktriangle}$	11.89 ± 0.11$^{\blacktriangle}$	10.66 ± 0.67$^{\blacktriangle}$	6.56 ± 1.77	3.93 ± 0.52$\Diamond$	27.71 ± 0.44$\Diamond$	16.96 ± 0.88	6.41 ± 0.79$\Diamond$
OleoH3P-ACBP	10.16 ± 0.04$^{\Delta}$	11.67 ± 0.37$^{\blacktriangle}$	12.86 ± 0.33$^{\blacktriangle}$	6.58 ± 0.94	2.61 ± 0.16$^{\blacktriangledown}$	28.81 ± 0.53$\Diamond$	18.06 ± 1.34	7.27 ± 0.73
ACBP-KDEL	12.98 ± 1.06$^{\blacktriangle}$	12.45 ± 0.87$^{\blacktriangle}$	11.86 ± 0.42$^{\blacktriangle}$	6.35 ± 0.67	3.84 ± 0.21$^{\blacktriangledown}$	23.25 ± 1.66$^{\blacktriangledown}$	20.12 ± 0.45	5.56 ± 0.57$^{\blacktriangledown}$
D9-ACBP-KDEL	10.33 ± 0.87$^{\Delta}$	12.30 ± 1.69$^{\blacktriangle}$	11.43 ± 1.36$^{\blacktriangle}$	7.01 ± 0.93	3.09 ± 0.15$^{\blacktriangledown}$	26.11 ± 4.98$^{\blacktriangledown}$	18.90 ± 2.04	5.65 ± 1.18$^{\blacktriangledown}$
ACBP	14.54 ± 0.43$^{\blacktriangle}$	10.51 ± 2.12	9.48 ± 0.38	7.23 ± 0.51	4.79 ± 0.77	18.28 ± 3.75$^{\blacktriangledown}$	23.27 ± 2.10$^{\blacktriangle}$	6.68 ± 0.83$\Diamond$
D9-ACBP	9.35 ± 0.68	6.61 ± 0.12	8.35 ± 0.87	6.82 ± 1.41	3.21 ± 0.15$^{\blacktriangledown}$	24.83 ± 3.01$^{\blacktriangledown}$	23.88 ± 0.63$^{\blacktriangle}$	11.78 ± 2.17$^{\blacktriangle}$
B82-Oleo-ACBP	9.91 ± 0.37$^{\blacktriangle}$	11.35 ± 0.03	12.23 ± 1.23$^{\blacktriangle}$	6.91 ± 0.55	2.94 ± 0.92$^{\blacktriangledown}$	29.21 ± 3.09	17.38 ± 1.92	5.93 ± 0.58$\Diamond$

Table 3.6. Acyl composition of the total acyl-CoA pool from *A. thaliana* T_3 developing seeds (15 DAF) transformed with *BnACBP* constructs. Mean %weight ± SD (n=4). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}/\Diamond$) values significantly greater/ smaller than WT at α=0.1.

Construct	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:1
WT_15DAF	8.19 ± 1.67	5.13 ± 0.67	7.39 ± 0.40	8.47 ± 0.35	6.18 ± 1.44	11.82 ± 0.84	32.19 ± 1.88	12.19 ± 1.08
ACBP-Oleo	17.54 ± 0.13$^{\blacktriangle}$	6.00 ± 0.02	7.84 ± 0.45	8.91 ± 0.53	4.28 ± 0.19$^{\blacktriangledown}$	10.45 ± 0.21	31.36 ± 0.83	7.62 ± 0.15$^{\blacktriangledown}$
OleoH3P-ACBP	13.40 ± 1.73$^{\blacktriangle}$	7.04 ± 0.67	7.16 ± 1.98	6.60 ± 1.25	3.19 ± 0.78$^{\blacktriangledown}$	17.01 ± 1.41$^{\blacktriangle}$	25.86 ± 1.83$^{\blacktriangledown}$	13.82 ± 1.59
ACBP-KDEL	17.05 ± 1.24$^{\blacktriangle}$	8.15 ± 1.56$^{\blacktriangle}$	9.43 ± 0.99	8.97 ± 1.53	4.01 ± 0.65$^{\blacktriangledown}$	12.81 ± 1.84	26.75 ± 0.72$^{\blacktriangledown}$	6.64 ± 1.07$^{\blacktriangledown}$
D9-ACBP-KDEL	11.80 ± 0.34$^{\blacktriangle}$	8.92 ± 0.31$^{\blacktriangle}$	8.58 ± 0.81	6.98 ± 0.24	3.28 ± 0.18$^{\blacktriangledown}$	19.39 ± 0.43$^{\blacktriangle}$	26.34 ± 0.52$^{\blacktriangledown}$	9.05 ± 0.48$^{\blacktriangledown}$
ACBP	21.75 ± 0.29$^{\blacktriangle}$	8.27 ± 0.66$^{\blacktriangle}$	7.85 ± 0.14	9.99 ± 0.81	5.03 ± 0.44	11.59 ± 0.67	20.62 ± 1.02$^{\blacktriangledown}$	4.79 ± 0.56$^{\blacktriangledown}$
D9-ACBP	15.93 ± 0.75$^{\blacktriangle}$	6.36 ± 1.26	6.59 ± 0.79	8.82 ± 2.01	3.63 ± 0.16$^{\blacktriangledown}$	12.01 ± 0.14	29.03 ± 1.14$^{\blacktriangledown}$	7.76 ± 0.65
B82-Oleo-ACBP	10.37 ± 1.19$^{\Delta}$	9.08 ± 1.72$^{\blacktriangle}$	11.08 ± 0.81$^{\blacktriangle}$	5.67 ± 0.58$^{\blacktriangledown}$	2.83 ± 0.29$^{\blacktriangledown}$	19.33 ± 3.74$^{\blacktriangle}$	27.20 ± 2.12$^{\blacktriangledown}$	9.81 ± 1.69

Table 3.7. Acyl composition of the total acyl-CoA pool from *A. thaliana* T_3 developing seeds (20 DAF) transformed with *BnACBP* constructs. Mean %weight ± SD (n=4). ($^{\blacktriangle}$/$^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}$/◊) values significantly greater/ smaller than WT at α=0.1.

Construct	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:1
WT_20DAF	14.07 ± 2.63	6.53 ± 0.24	11.39 ± 1.38	13.61 ± 3.69	4.26 ± 0.84	6.74 ± 0.79	21.57 ± 4.09	6.14 ± 1.00
ACBP-Oleo	24.94 ± 2.51$^{\blacktriangle}$	6.07 ± 0.77	9.33 ± 3.23	18.57 ± 4.57	6.52 ± 0.92$^{\blacktriangle}$	7.01 ± 2.60	18.05 ± 6.07	4.72 ± 1.30
OleoH3P-ACBP	20.69 ± 1.89$^{\Delta}$	6.46 ± 0.27	6.21 ± 0.67$^{\blacktriangledown}$	15.56 ± 0.40	5.53 ± 1.32	12.46 ± 0.17$^{\blacktriangle}$	19.87 ± 0.29	7.93 ± 1.54
ACBP-KDEL	27.52 ± 0.91$^{\blacktriangle}$	7.21 ± 0.18	9.08 ± 0.76	24.74 ± 1.10$^{\blacktriangle}$	6.97 ± 0.16$^{\blacktriangle}$	4.85 ± 0.71	11.43 ± 0.39$^{\blacktriangledown}$	2.55 ± 0.52$^{\blacktriangledown}$
D9-ACBP-KDEL	16.92 ± 0.18	7.71 ± 0.23	7.66 ± 0.19	9.60 ± 0.35	2.81 ± 0.24	8.85 ± 1.46	28.96 ± 1.26	6.07 ± 1.39
ACBP	30.50 ± 0.54$^{\blacktriangle}$	8.22 ± 0.35	7.41 ± 0.03	19.65 ± 1.28$^{\Delta}$	5.55 ± 0.38	5.12 ± 0.79	13.23 ± 0.25◊	1.59 ± 0.21$^{\blacktriangledown}$
D9-ACBP	22.87 ± 1.36$^{\blacktriangle}$	6.87 ± 1.02	10.17 ± 1.61	23.31 ± 0.64$^{\Delta}$	4.52 ± 0.31	3.84 ± 1.80	18.85 ± 1.87	3.32 ± 1.18$^{\blacktriangledown}$
B82-Oleo-ACBP	26.02 ± 3.99$^{\blacktriangle}$	7.71 ± 0.23	6.64 ± 0.28	18.28 ± 4.56	5.04 ± 1.29	10.08 ± 0.32	18.67 ± 6.03	4.55 ± 2.59

3.3.6 Analysis of the fatty acid composition and content of seed oil from *B. napus* T_1 seeds expressing *ACBP-Oleo* construct.

B. napus transformation with pGreen-based vectors harbouring *BnACBP* constructs has proven challenging. Only one transgenic line with ACBP-Oleo construct has been produced. Seed oil was extracted from mature seeds of *B. napus* ACBP-Oleo T_1, Escape line and WT in three technical replicates. Based on two analytical methods, gravimetry and GC, it was determined that transgenic seeds had significantly higher seed oil content compared to Escape line and WT seeds (Table 3.8).

FA composition of T_1 seeds was compared to the Escape line used as a control. As described for *A. thaliana* seeds expressing BnACBP constructs, *B. napus* transgenic seeds oil accumulated a higher proportion of PUFA, primarily at the expense of MUFA compared to Escape line (Table 3.9). Both major PUFA in *B. napus* seed oil, 18:2 and 18:3, increased in T_1 seeds while 18:1 decreased (Table 3.10). In contrast to *A. thaliana*, canola seed oil has only minor amounts of VLC-FA (20:0 and 20:1), and thus the increase in PUFA appears to occur not at the expense of 18:1 elongation but rather due to a more efficient channelling of 18:1 towards desaturation. Statistical analysis performed on mol% values gave similar results as the analysis with %weight (Appendix 2).

Table 3.8. Seed oil content of *B. napus* T_1 line determined by gravimetric method (Grav) and by GC. Mean %weight ± SD (n=3). (▲) values significantly greater than WT at α=0.05; (*) values significantly greater than Escape line at α=0.05.

Construct	Oil% (Grav), %weight	Oil% (GC), %weight	Oil/seed (Grav), mg	Oil/see (GC), mg
WT	32.54 ± 0.88	27.33 ± 0.95	1.17 ± 0.04	0.98 ± 0.03
Escape	32.21 ± 1.36	27.57 ± 1.27	1.12 ± 0.07	0.96 ± 0.07
ACBP-Oleo	34.89 ± 0.50▲*	30.82 ± 0.79▲*	1.44 ± 0.02▲*	1.27 ± 0.05▲*

Table 3.9. Composition of FA classes in *B. napus* T_1 seeds. Mean %weight ± SD (n=3, technical replicates). (▲/▼) values significantly greater/ smaller than Escape line at α=0.05.

Construct	SFA	MUFA	PUFA
Escape	7.46 ± 0.11	61.25 ± 0.06	31.23 ± 0.09
ACBP-Oleo	6.91 ± 0.07▼	59.46 ± 0.90▼	33.59 ± 0.98▲

Table 3.10. FA composition of the seed oil from *B. napus* T_1 seeds. Mean %weight ± SD (n=3, technical replicates of each). (▲/▼) values significantly greater/ smaller than Escape line at α=0.05.

Construct	16:0	18:0	18:1	18:2	18:3	20:0	20:1
Escape	4.66 ± 0.07	1.66 ± 0.05	57.02 ± 0.06	18.84 ± 0.05	12.39 ± 0.13	0.67 ± 0.005	1.44 ± 0.02
ACBP-Oleo	4.45 ± 0.05	1.44 ± 0.04	55.09 ± 0.96▼	20.13 ± 0.64▲	13.46 ± 0.35▲	0.60 ± 0.02	1.40 ± 0.04

3.4 Discussion

Small cytosolic ACBPs have been found in a number of higher plants, and are believed to perform a housekeeping function in all cells by maintaining the acyl-CoA pool and facilitating intracellular transport of acyl-CoAs. ACBP can also acquire more specialized functions in metabolic processes involving acyl-CoAs. In *A. thaliana,* 10 kDa ACBP has been demonstrated to play a role in mediating freezing stress responses associated with phospholipid metabolism (Chen *et al.*, 2008). ACBP levels were found to be the highest in developing seeds of *A. thaliana* and *B. napus* compared to other tissues indicating that this protein might have a specialized role in seed development and maturation (Engeseth *et al.*, 1996)

Overexpression of *ACBP* in *A. thaliana* developing seeds resulted in increased amounts of PUFA in seed oil. The elevated percentage of PUFA in seed oil was observed primarily due to an increase in 18:2 at the expense of 20:1. Also, most of the transgenic lines had a reduction in 18:0 and 18:3 in seed oil. These results agree with the observations made by Enikeev and Mishutina (2005) while studying the effect of the genetic vector structure on the physiological effect of the plant transformation. In their experiment, cDNA encoding the cytosolic ACBP from *A. thaliana* was expressed in *B. napus* in sense and antisense orientation under a 35S promoter. In a high erucic acid cultivar transformed with sense ACBP construct, decreased levels of long chain MUFA (20:1 and 22:1) were documented, while lines expressing antisense ACBP constructs had exhibited a 1.5 – 2-fold increase in the 22:1 content of the seed oil. These data support our

findings that ACBP levels in developing seeds might have important role in partitioning of acyl-CoA between FA elongation and desaturation.

In order to investigate if the changes in FA composition of seed oil were due to the changes in the acyl-CoA pool or due to acyl-CoA independent mechanisms, the composition of the acyl-CoA pool in transgenic seeds was analyzed at different developmental stages. An increase in the proportion of saturated and some unsaturated (C16:0, C18:0 and C18:2) acyl groups at the expense of very-long chain saturated and monounsaturated groups (C20:0, C20:1 and C22:1) suggests that the changes in the FA composition of seed oil might be a direct effect of changes in composition of the acyl-CoA pool. Increases in the contribution of saturated acyl-CoAs to the total acyl-CoA pool have also been observed in yeast and mice over-expressing ACBP (Mandrup S. *et al.*, 1993; Huang *et al.*, 2005). In the plant system, this could be explained by premature release of the acyl groups from plastids and conversion to acyl-CoA by acyl-CoA synthetase activity, enhanced due to the product removal by ACBP (Mandrup S. *et al.*, 1993). The increase in C18:2 can be explained by more efficient acyl editing of the membrane phospholipids in the presence of higher ACBP levels (Huang *et al.*, 2005; Chapter 4).

Results of the protein analysis of the developing and mature T_3 seeds suggest that levels of the transgenic ACBP were affected by the fusion partner. The highest levels of ACBP-Oleosin and OleosinH3P-ACBP products could be explained by the presence of the oleosin fusion partner which normally accumulates in developing seeds and remains stable even upon the desiccation of

the seed (Murphy *et al.*, 1989). On the contrary, relatively low levels of the cytosolic and KDEL-fusion constructs' products in developing seeds might be a result of the natural protein turnover in the cell.

ACBP-Oleosin and OleosinH3P-ACBP fusions with GFP were targeted to OB and were stably accumulated in mature seeds. On the other hand, ACBP-KDEL appeared to be located on membrane structures within the cell, which might have facilitated a close proximity of these binding sites to the acyltransferases located on the ER. This could explain the effect of this construct on seed oil composition comparable to constructs with oleosin, even though the levels of ACBP-KDEL were much lower than ACBP fusions with oleosin. In the case of the ACBP construct that was expressed without the addition of targeting sequences, the recombinant protein was found in the cytosol and nucleus (which is an artifact). This construct closely reflects the structure and localization of the native ACBP, and despite relatively low levels of expression, had significant and distinctive effects on the acyl-CoA pool and seed oil composition. It appears that a combination of absolute levels and cellular localization of the transgenic ACBP in developing seeds may influence the effect on seed oil.

Based on these observations we propose that the increase in the concentration of acyl-CoA binding sites in cytosol affected the partitioning of 18:1-CoA between desaturation and elongation. A possible mechanism of ACBP enhancing 18:2 levels at the expense of 20:1 in seed oil as a function of increased concentration of acyl-CoA binding sites is illustrated on the Figure 3.10.

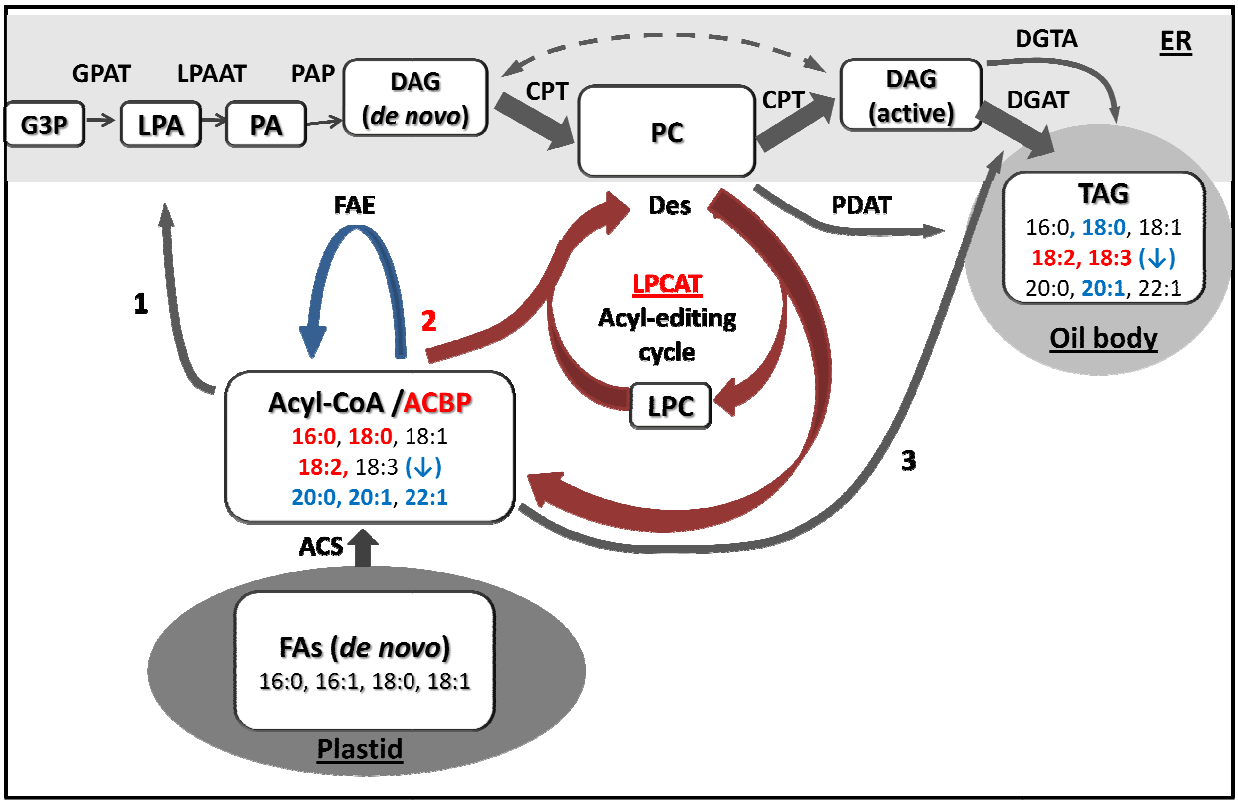


Figure 3.10. Proposed model of the effect of increased levels of cytosolic ACBP on acyl channelling in seed oil biosynthesis. Acyl groups with decreased or increased content in acyl-CoA pool and in TAG in *A. thaliana* seeds transformed with *BnACBP* are highlighted in blue or red, respectively. Arrows in brackets represent the changes in *A. thaliana* seeds transformed with *BnACBP* fusion with oleosin variants. Increased content of ACBP in cytosol may affect acyl editing (2) by stimulating the forward and reverse reactions of LPCAT (red thick arrows), and releasing polyunsaturated acyl chains into the acyl-CoA pool, where they are used by acyltransferases and incorporated into TAG (1 and 2). Increased channelling of the *de novo* FAs towards desaturation on PC is done at the expense of the elongation (blue arrow), which resulted in the decreased proportions of VLC-FAs in acyl-CoA pool and in TAG. (Names of the enzymes and lipid classes are the same as in Figure 2.2)

The ratio of the 18:1-CoA channelling towards elongation and desaturation shifts in favour of the later, possibly by enhancing the activity of phospholipid acyltransferase(s) in the presence of higher levels of ACBP. This in turn can affect the overall turnover rate of the acyl groups (acyl editing) on phospholipids (particularly on PC) enhancing channelling of the desaturation products (18:2 and 18:3) back to acyl-CoA pool or directly to neutral lipids. Further experiments including *in vitro* studies of the recombinant BnACBP and its effect on the acyltransferases will provide more insight into the possible mechanisms of ACBP affecting seed oil biosynthesis.

4. Biochemical studies with recombinant BnACBP

4.1 Introduction

The main property of acyl-CoA binding proteins (ACBP) is the ability to bind acyl-CoA with high affinity. The binding stoichiometry between ACBP and the ligand is approximately 1 mol/mol (Rasmussen *et al.*, 1990). Both the acyl chain and the CoA head group of acyl-CoA are involved in binding of acyl-CoA to ACBP, which makes medium to long-chain acyl-CoAs an exclusive ligand for ACBP (Rosendal *et al.*, 1993). The binding affinity of acyl-CoA esters for ACBP have been shown to be strongly dependent on the length of the acyl chain and the number of double bounds, and can vary between species and isoforms (Faergeman *et al.*, 1996). It has been demonstrated through binding studies that ACBPs have different affinities toward the most common acyl-CoAs found in plants, suggesting that certain acyl-CoA species may be preferred over the others for binding and transport by ACBP (Brown *et al.*, 1998). It is also possible that different ACBP isoforms have different binding specificities. For example, it has been shown that *B. napus* recombinant ACBP (rBnACBP) had a higher affinity towards oleoyl-CoA (18:1-CoA) than palmitoyl-CoA (16:0-CoA) (Brown *et al.*, 1998).

Lipidex-1000 binding assays demonstrated strong binding of rAthACBP with [^{14}C]16:0-CoA at low micromolar concentrations (Engeseth *et al.*, 1996). The use of titration microcalorimetry revealed that interaction between the acyl-CoA ligand and the plant ACBP is greatly affected by the acyl-chain length and the presence and number of double bonds in acyl-CoA (Pacovsky, 1996). Binding

specificities of the *A. thaliana* ACBP family proteins is outlined in the Table 2.2. The differential preference for different ligands in combination with the knowledge of the subcellular localization and tissue expression, may point out some possible functions of different ACBPs.

A number of *in vitro* experiments have shown that ACBP can modulate the activity of the enzymes involved in lipid metabolism (Reviewed in Faergeman *et al.*, 2007). In one of the early studies of the ACBP effect on glycerolipid synthesis, Rasmussen *et al.* (1993) observed that incubation of rabbit mammary-gland microsomes with bovine L-ACBP decreased the incorporation of [^{14}C]16:0-CoA into triacylglycerol (TAG), but did not affect the synthesis of phospholipids significantly. Thus, the authors suggested that ACBP could compete with the triacylglycerol-synthesizing pathways, but not with the phospholipid-synthesizing enzymes, for acyl-CoA esters. The purified human liver ACBP increased the rate of PC formation from arachidonoyl-CoA by acyl-CoA: lysophospholipid acyltransferase (LAT) in the human red blood cell membranes (Fyrst *et al.*, 1995). Also, it was found that ACBP-bound acyl-CoA was preferentially utilized over free cytosolic acyl-CoA as a substrate by LAT.

Lysophosphatidylcholine acyltransferase (LPCAT) is one of the most active enzymes in seed oil metabolism and is postulated to be involved in acyl exchange between acyl-CoA and phosphatidylcholine (PC) metabolic pools (Stymne and Stobart, 1984). These authors demonstrated that the reverse reaction of LPCAT (return of the acyl group from *sn*-2 position of PC back into acyl-CoA pool) was stimulated by bovine serum albumin (BSA) *in vitro*, which can also bind acyl-

CoAs. ACBP has also been shown to modulate activity and specificity of acyltransferases involved in phosphatidic acid (PA) biosynthesis (Jolly *et al.*, 2000; Kannan *et al.*, 2003). rBnACBP increased *sn*-glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT) activities in a manner dependent on ACBP: acyl-CoA ratio in the reaction mixture (Brown *et al.*, 1998; Brown *et al.*, 2002). Recombinant rat liver ACBP stimulated microsomal incorporation of oleoyl-CoA and arachidonoyl-CoA by GPAT (Jolly *et al.*, 2000). Overexpression of the liver ACBP in mouse resulted in a 4-fold increase of the liver microsomal GPAT activity (Huang *et al.*, 2005).

The acyl-CoA: cholesterol acyltransferase (ACAT) activity was strongly inhibited in microsomes from human mononuclear phagocytes by bovine ACBP at different molar ratio of ACBP and acyl-CoA (Kerkhoff *et al.*, 1997). Mouse recombinant ACBP also inhibited ACAT in rat liver microsomes in the absence of exogenous cholesterol, but significantly stimulated ACAT activity when exogenous cholesterol was provided (Chao *et al.*, 2003). Also, ACBP: acyl-CoA complex was significantly more effective than acyl-CoA alone for initiating the microsomal ACAT reaction. Animal ACAT is homologous to animal and plant diacylglycerol acyltransferase 1 (DGAT1) (Cases *et al.*, 1998; Hobbs *et al.*, 1999), thus, there is a reason to expect that ACBP might have an effect on DGAT1 activity in developing seeds.

In this study, we performed *in vitro* assays using yeast and plant cell-free systems to study the effect of the rBnACBP on the acyltransferases involved in seed oil formation: GPAT, DGAT and LPCAT. GPAT, which catalyzes the first

committed step in the Kennedy pathway, and has already been demonstrated to be affected by ACBP *in vitro* studies with microsomes from *B. napus* developing embryos (Brown *et al*., 1998). DGAT, an enzyme performing the final acylation of the TAG (Ramli *et al.*, 2002), is considered to have a substantial effect on the flow of carbon into seed oil has been stimulated by BSA *in vitro* (Little *et al*., 1994). LPCAT was chosen for *in vitro* studies based on our results of the ACBP overexpression in *A. thaliana* developing seeds. The increase in PUFAs in seed oil from the transgenic lines suggested that acyl exchange between acyl-CoA and the PC pool mediated by LPCAT activity might have been affected.

4.2 Experimental procedures

4.2.1 Expression of the recombinant *B. napus* ACBP in *E. coli*

Glutathione S-transferase (GST) Gene Fusion System (GE Healthcare, Baie d'Urfé, QC, Canada) was used for expression and purification of the recombinant *B. napus* ACBP (rBnACBP) in *Eschericia coli*. *BnACBP* cDNA (X77134) was amplified using 2422 and 2423 primers and cloned into pGEX-4T1 expression vector using *BamHI* and *XhoI* restriction endonuclease sites, resulting in the expression vector pGEX-BnACBP (Figure 4.1). BL21(DE3) *E. coli* transformed with pGEX-BnACBP was grown in LB medium with IPTG for 12 hours. GST-BnACBP fusion protein was purified from the *E. coli* culture lysate using Glutathione Sepharose 4B (GE Healthcare, Baie d'Urfe, QC, Canada) column. The GST tag was removed from GST-rBnACBP by treatment with thrombin protease.

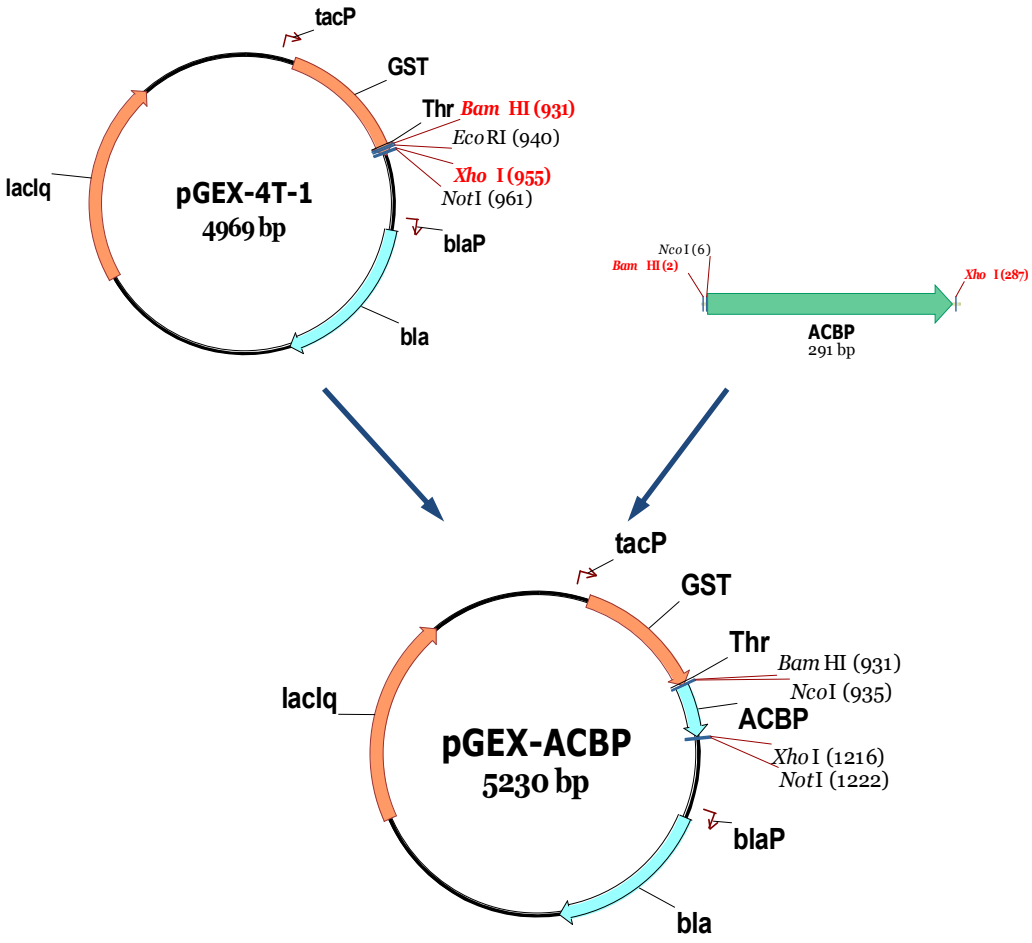


Figure 4.1 Schematic drawing of the molecular cloning of pGEX-ACBP expression vector for *E. coli* transformation. Components of the vector: ACBP – *B. napus ACBP* cDNA; GST – Glutathione S-transferase gene; tacP – *tac* promoter; Thr – thrombin recognition site; bla – Beta-lactamase gene; blaP – *bla* promoter; lacIq – *lac* repressor gene.

The concentration of the recombinant protein was determined using the Bio-Rad protein microassay (Bio-Rad, Missisauga, ON, Canada) based on the Bradford M. (1976) procedure, with BSA as a standard.The purity of rBnACBP was analyzed by SDS-PAGE using standard protocols (Sambrook *et al.*, 1989) and stained with Coomassie Brilliant Blue R 250 or blotted for Western analysis. Blotted samples were probed with polyclonal antibody directed against *B. napus* ACBP (Brown *et al.*, 1998). ACBP was detected using secondary donkey anti-rabbit IRDye® 800 CW (LiCor Biosciences, Lincoln, NE, USA) and analyzed using the Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE, USA).

4.2.2 Lipidex-1000 binding assay

The binding assay for examining the interaction of radiolabeled acyl-CoAs with rBnACBP was performed using Lipidex-1000 (Packard BioScience Groningen, The Netherlands) as described in Zeng *et al.* (2006) with minor modifications. Briefly, 40 pmol of rBnACBP were incubated with one radiolabeled acyl-CoA (0.1 – 1 μM) at a time in 200 μL binding buffer (10 mM potassium phosphate, pH 7.4) at 30°C for 30 min. The mixture was then placed on ice for 10 min, and 400 μL of ice-cold 50% (v/v) Lipidex-1000 slurry in binding buffer were added. Samples were incubated on ice with occasional mixing for 20 min, and then centrifuged at 12000 ***g*** for 5 min at 4°C. Two hundred microliters of the supernatant from each sample were combined with 5 ml of Ecolite^{TM}(+) scintillant (MP Biochemicals, Irvine, CA, USA) and analyzed for radioactivity in a scintillation counter (Beckman Coulter, LS-6500, Mississauga, ON, Canada).

All assays were done in three replicates and control samples with no protein added were used to correct for the amount of acyl-CoA that was not absorbed by Lipidex-1000 beads.

4.2.3 Yeast strains and growth conditions

Yeast BY4742 strain *LPCAT* knock-out ΔYOR175c was transformed with the expression vectors harboring *AthLPCAT-1* or *AthLPCAT-2* cDNA, pYES2/NT C-At1g12640 and pYES2/NT C-At1g63050, respectively. Yeast strain H1246Y, which is a quadruple knockout (*are-1*Δ *are-2*Δ *dga1*Δ *lro1*Δ) deprived of TAG biosynthesis, was transformed with pYES2.1-TOPO-BnDGAT1. Yeast GPAT knock-out (*gat1*Δ) of BY4742 strain was transformed with pYES2.1-TOPO-BnGPAT4. All yeast strains used were also transformed with pYES2.1-TOPO-*lacZ,* which was used as a control. Yeast cultures were inoculated in liquid 2% glucose synthetic medium lacking uracil and grown on a rotary shaker at 30°C to early stationary phase (A_{600} 8-10). Cells were harvested by centrifugation at 2500 ***g*** for 7 min at 4°C and washed once with the induction buffer (SC-U, 2% galactose). Cells were resuspended in 50 mL of induction medium to obtain A_{600} 0.4 and grown on a rotary shaker for 20-22 h before harvesting for the microsome preparation.

4.2.4 Preparation of microsomes

Yeast cultures were centrifuged at 2500 ***g*** for 7 min at 4°C, washed once with distilled water and resuspended in ice-cold 50 mM Tris-HCl, pH 8.0 buffer, containing 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol. To each sample,

approximately the same vol of 0.5 mm Zirconia/Silica beads (BioSpec Products Inc., Bartlesville, OK) was added, and the cells were disrupted in a bead beater (BioSpec Products Inc.) by shaking 3 x 1 min intervened by 1-2 min cooling on ice. The extracts were centrifuged at 2500 ***g*** for 5 min at 4°C and the supernatant was subjected to ultracentrifugation at 100000 ***g*** for 1 h at 4°C. The pellet was resuspended in 10 mM Hepes-NaOH, pH 7.4 buffer, frozen in liquid N_2 and stored at -80°C.

The microspore-derived (MD) cell suspension culture of (Orr *et al.*, 1986)*B. napus* L. cv Jet Neuf was maintained according to Orr *et al.* (1986). Harvested cells were washed on a 60 μM nylon mesh, blotted dry, frozen in liquid N_2 and stored at -80°C. Frozen tissue was thawed on ice and ground in 4 vol of grinding buffer (0.2 M Hepes-NaOH, pH 7.4 0.5 M sucrose) with a PowerGen700 grinder (Fisher Scientific, Whitby, ON, Canada). The homogenate was centrifuged at 10000 ***g*** for 30 min at 4°C and the resulting pellet discarded. The supernatant was filtered through cheese cloth and centrifuged at 100000 ***g*** for 1 h at 4°C. The resulting pellet was resuspended in 10 mM Hepes-NaOH, pH 7.4 buffer, frozen in liquid N_2 and stored at -80°C. One milliliter of resuspension buffer was used for every 10 g of original tissue weight. The protein concentration of the microsomal extracts was determined using the same method used for rBnACBP.

4.2.5 LPCAT assays

LPCAT (forward reaction) assay conditions were modified from Furukawa-Stoffer *et al.* (2003). The microsomal fraction from ΔYOR175c yeast expressing *AthLPCAT-1* (~25-50 μg) was incubated in 80 mM Tris-HCl, pH 7.5 buffer,

containing 0.21 M sorbitol, 0.13 mM EDTA, 15 μM [1-^{14}C]18:1-CoA (5.7 mCi/mmol, GE Healthcare, Baie d'Urfé, QC, Canada), 75 μM 18:1-LPC (Avanti Polar Lipids, Inc., Alabaster, AL, USA) and varying amounts of rBnACBP or BSA (5-25 μM) in the total volume of 50 μL for 10 min at 30°C. The reaction was terminated by addition of 2 mL of chloroform/methanol (2:1, v/v). Phase separation was induced by the addition of 1 mL 0.9% (w/v) KCl. Following brief centrifugation, the lower organic phase was transferred to a clean glass tube. Lipid residues in the aqueous phase were re-extracted with 1 mL chloroform, and combined with the first extract of the organic phase. The solvent was dried under a gentle stream of N_2 gas at 37°C. Samples were re-suspended in 70 μL chloroform and applied in 2.2 cm lanes to a 20x20 cm pre-coated TLC plate SIL G-25. A control lane with 5 μL of 100 μM *sn*1-16:0, *sn*2-[1-^{14}C]18:1-PC (56 mCi/mmol, GE Healthcare, Baie d'Urfé, QC, Canada) was used to confirm the identity of the radioactive product. The TLC plate was developed in a chloroform/methanol/acetic acid/water (85: 15: 10: 3.5, by vol.) solvent system. PC product was visualized by a phosphoimaging system (Typhoon Trio Variable Mode Imager, GE Healthcare, Baie d'Urfe, QC, Canada). TLC spots corresponding to the position of PC product were scraped from the plate, combined with 5 ml of Ecolite^{TM}(+) scintillant and analyzed for radioactivity.

Acylation of PC in the absence of added LPC in microsomes from ΔYOR175c yeast expressing *AthLPCAT-2* or *B. napus* MD cell suspension cultures was assayed using the same protocol described above but with the omission of 18:1-LPC.

The protocol for assaying the LPCAT reverse reaction was modified from Stymne and Glad (1981). Microsomes from ΔYOR175c yeast expressing *AthLPCAT-1* or *AthLPCAT-2* (~15 μg) were lyophilized and washed with benzene containing 5 nmol *sn*1-16:0-sn2-[1-^{14}C]18:2-PC (5.17 mCi/mmol, GE Healthcare, Baie d'Urfé, QC, Canada). Benzene was immediately evaporated under a gentle stream of N_2 gas and 100 μL of the assay medium were added. The reaction medium consisted of 50 μM 18:1-CoA (Sigma, Oakville, ON), 2 mM CoA (Sigma, Oakville, ON, Canada) and varying amounts of rBnACBP or BSA (25-75 μM) in 0.1 mM phosphate buffer, pH 7.2. The non-radioactive 18:1-CoA was added in order to trap [1-^{14}C]18:2-CoA formed by the reverse reaction of LPCAT, which otherwise would be directly re-acylated to the simultaneously formed LPC. The reaction was allowed to proceed for 30 min at 30°C and was terminated with 375 μL of chloroform/methanol (1:2, v/v). Extraction of the chloroform soluble lipids from the reaction mixture was performed according to a standard procedure (Bligh and Dyer, 1959). The volume of the aqueous fraction was reduced to approximately 50 μL under vacuum (SpeedVac with Vapor Net AES2000, Savant) and was then applied to a 20x20 cm pre-coated TLC plate SIL G-25. The TLC plate was developed in a methyl acetate/ *n*-propanol/ chloroform/ methanol/ 0.25% KCl in water (25: 25: 25: 10: 9, by vol) solvent system. Acyl-CoAs were visualized using phosphoimaging, removed from the TLC plate and combined with 5 mL of Ecolite^{TM}(+) scintillant for analysis using the liquid scintillation counter.

4.2.6 DGAT assay

The DGAT assay was performed following the procedure described in Little *et al.* (1994) with slight modifications. The reaction mixture consisted of 0.2 M HEPES/NaOH pH 7.4 buffer, containing 3 mM $MgCl_2$, 330 µM *sn*-1,2-diolein, 0.02% (v/v) Tween-20, 15 µM [^{14}C]18:1-CoA (5.7 mCi/mmol) and varying amounts of rBnACBP or BSA. The assay was initiated with the addition of microsomal fraction from *B. napus* MD cell suspension cultures, *B. napus* developing embryos or from H1246 yeast producing BnDGAT1 (~50µg). The reaction conducted in 60 µL of total volume was incubated for 10 min at 30°C and was terminated by addition of 10 µL of 10% (w/v) SDS. Aliquots of 50 µL were applied on the TLC plate and left to dry at room temperature. A control lane with 25 µL of triolein (30 mg/ml in chloroform) was used to establish the migration distance of TAG. The plate was developed with hexane/diethyl ether (4:1, v/v). Based on the TAG control, visualized with iodine vapour, corresponding spots in the adjacent lanes containing the reaction mixtures were scraped from the plate and radioactivity was determined in 5 mL scintillant. Enzyme assays were performed in triplicates.

4.2.7 GPAT assay

GPAT assays were performed *in vitro* following the protocols described in Zheng and Zo, (2001) and Zheng *et al.* (2003) with some modifications. The reaction was conducted in 50 µl of Hepes buffer (40 mM, pH 7.2) with 50 µM [U-^{14}C]G3P (30 mCi/mmol), 15 µM 18:1-CoA, 1 mM DTT, 5 mM EDTA and varying amounts of BSA or rBnACBP (0-25 µM). Reactions were initiated by

addition of the microsomal protein (~50μg) and incubated at 30°C for 10 min. The assay was stopped by addition of 2 mL of chloroform/methanol (2:1, v/v). Following the addition of 1 mL of 1M KCl in 0.2M H_3PO_4 and brief centrifugation, the lower organic phase was collected in a clean glass tube and dried under a gentle stream of N_2 gas at 37°C. Samples were re-suspended in 70 μL chloroform and applied in 2.2 cm lanes to a 20x20 cm pre-coated TLC plate SIL G-25. The TLC plate was developed in a chloroform/methanol/acetic acid/5% (w/v) $NaHSO_3$ in water (100: 40: 13: 4, by vol.) solvent system. Radioactive products were visualized by a phosphoimaging system (Typhoon Trio Variable Mode Imager). TLC spots corresponding to the position of LPA, PA, DAG were scraped from the plate, combined with 5 ml of Ecolite^{TM}(+) scintillant and analyzed for radioactivity.

4.2.8 Cloning of *B. napus* ACBP variants

BnACBP cDNA sequence (Accession number X77134) was used as a query to search the *Brassica* EST database for similar sequences. Based on the sequence of the 5' and 3' ends of the EST found, primers (MT-F1, MT-B1, MT-B2 and MT-B3, Table A1.1) were designed to amplify three cDNA variants of *BnACBP* from *B. napus* cDNA library. PCR products (*BnACBP-1*, *-2* and *-3*) were cloned into pCR4 vectors and sequenced using ABI BigDye Terminator v3.1 Cycle Sequencing Kit. Samples were analyzed in an automatic sequencer (Applied Biosystems 3730 DNA Analyzer) at the Molecular Biology Facility at the University of Alberta.

4.3 Results

4.3.1 Recombinant BnACBP binds acyl-CoAs *in vitro*

We expressed and purified recombinant *B. napus* cytosolic ACBP in *E. coli* as a GST-fusion protein (Figure 4.2a). The GST tag was cleaved off with thrombin protease resulting in 95-99% pure rBnACBP. The only impurities that remained in rBnACBP sample were GST-rBnACBP fusion protein and cleaved GST. The identity of the recombinant protein was confirmed by Western blot analysis with polyclonal antibodies raised against rBnACBP (Figure 4.2b). The ability of rBnACBP to bind acyl-CoA was confirmed using the Lipidex-1000 binding assay (Figure 4.3). Substantial binding of 18:1- and 18:2-CoA occurred at low concentrations (< 1 μM) of total acyl-CoA while the binding of α-18:3-CoA was considerably weaker. The interaction of 18:1-CoA with rBnACBP was similar to that observed by Brown *et al.* (1998).

4.3.2 Recombinant BnACBP stimulates AthLPCAT activity *in vitro*

We hypothesized that the changes in FA composition of seed oil from developing seeds overexpressing *BnACBP* might be due to an increase in cytosolic ACBP affecting the partitioning of 18:1-CoA between desaturation and elongation pathways. Since desaturation of 18:1 to 18:2, and subsequently to α-18:3, takes place on PC, we set out to determine the effect of soluble ACBP on LPCAT activity, which, in the forward reaction, catalyzes the acyl-CoA dependent acylation of the *sn*-2 position of lysophosphatidylcholine (LPC) to form PC.

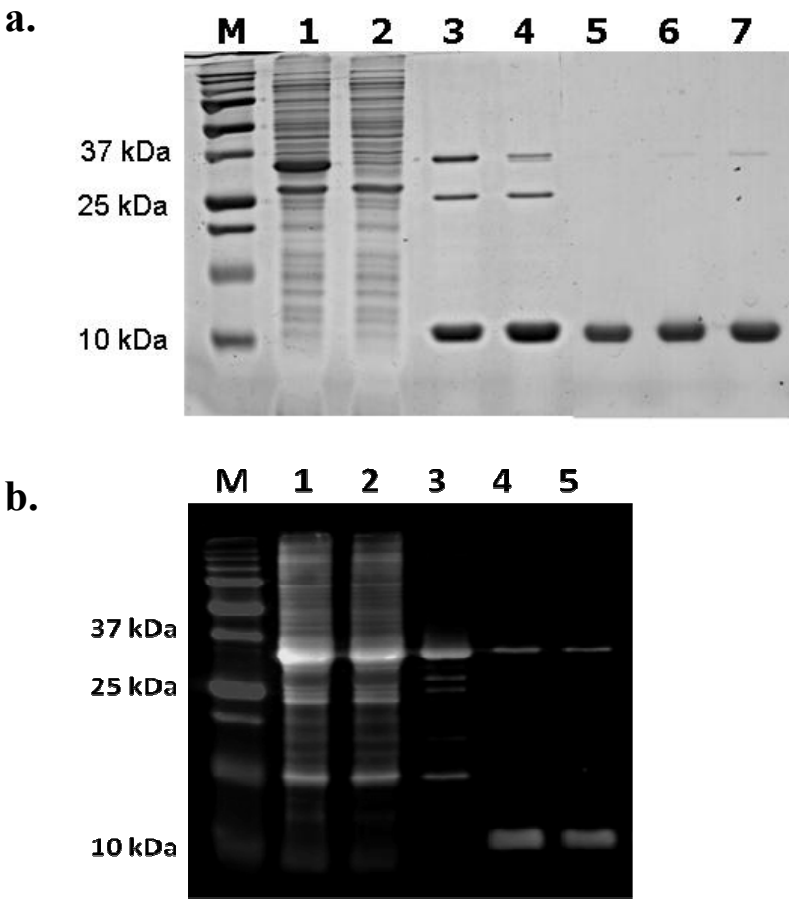


Figure 4.2. Analysis of rBnACBP following gel electrophoresis of *E. coli* sonicate and fractions obtained during purification. (a) Analysis of rBnACBP on 15% SDS-PAGE stained with Coomassie dye. Lane M: protein markers (Precision Plus Protein™ Standards, Bio-Rad); lane 1: *E.coli* lysate (~ 15 µg); lane 2: eluate of the sonicate from the Glutathione Sepharose 4B column; lanes 3-4: eluate of rBnACBP cleaved from GST with thrombin (~ 2.5 µg); lanes 5-7 – rBnACBP re-eluted through the column (~ 1.5 µg). (b) Western blot of rBnACBP on 15% SDS-PAGE incubated with polyclonal antibodies against BnACBP. Lane M: Protein markers (Precision Plus Protein™ Standards, Bio-Rad); lane 1: *E.coli* sonicate; lane 2: *E.coli* total soluble protein; lane 3: GSB-rBnACBP fusion protein; lanes 4-5: eluate of rBnACBP cleaved from GST with thrombin.

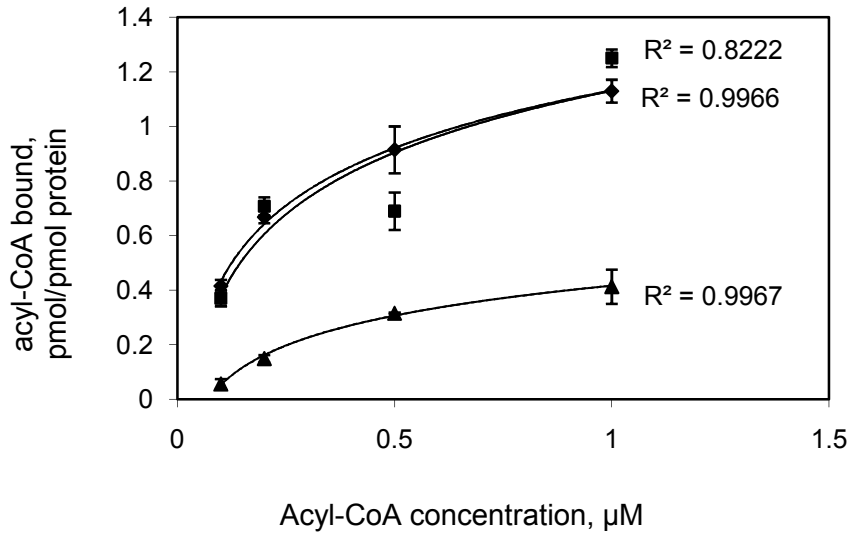


Figure 4.3 Lipidex-1000 binding assay of rBnACBP with [1-^{14}C]18:1-CoA (♦), [1-^{14}C]18:2-CoA (■) or [1-^{14}C]18:3-CoA (▲); (R-squared values for logarithmic trend).

The yeast LPCAT knock-out mutant ΔYOR175c was transformed with the recently cloned cDNAs encoding *A. thaliana* LPCAT isoforms (Ståhl *et al.*, 2008). Microsomal fraction from transformed yeast was incubated with [1-^{14}C]18:1-CoA and 18:1-LPC substrates. rBnACBP or BSA was added to the reaction mixture in various ratios to [1-^{14}C]18:1-CoA . BSA is known to bind medium to long chain acyl-CoA esters (Richards *et al.*, 1990), and was included in the experiment as a control. For the purposes of this discussion, the ratio of binding protein to acyl-CoA shall be referred to as "R".

rBnACBP increased AthLPCAT activity in yeast microsomes by 40% *in vitro* when R reached 1 and, thereafter, AthLPCAT activity was not affected significantly by further addition of binding protein (Figure 4.4). BSA appeared to have no effect on the amount of [1-^{14}C]PC produced at low concentrations of BSA, but at R from 0.66 to 1.66, AthLPCAT activity decreased by 23%. The inhibitory effect of BSA on AthLPCAT on the forward reaction was even more pronounced when higher concentrations of acyl-CoA were used in the reaction mixture (Figure 4.5).

The incorporation of [1-^{14}C]18:1 from the acyl-CoA pool into PC was studied in microsomes from yeast and MD cell suspension cultures of *B. napus* L. cv Jet Neuf under conditions where no exogenous LPC was provided. These MD cell suspension cultures have previously been used for studies of TAG biosynthesis and acyltransferase action (Weselake and Taylor, 1999; Furukawa-Stoffer *et al*., 2003). In the microsomal fraction of yeast expressing *AthLPCAT*, BSA or rBnACBP stimulated the synthesis of [1-^{14}C]PC (Figure 4.6a).

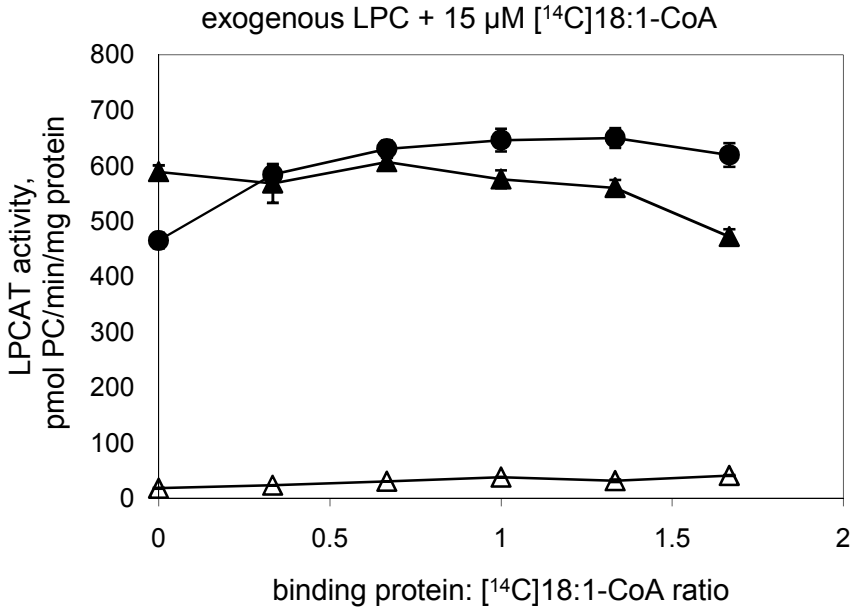


Figure 4.4 Effect of rBnACBP (circles) or BSA (triangles) on LPCAT activity in the presence of added LPC. [1-^{14}C]18:1-CoA (15 µM) and LPC (75 µM) were incubated with microsomes (~50 µg) from ΔYOR175c yeast transformed with *AthLPCAT-1* (filled markers) or lacZ (open markers) (n=3, mean ± SE).

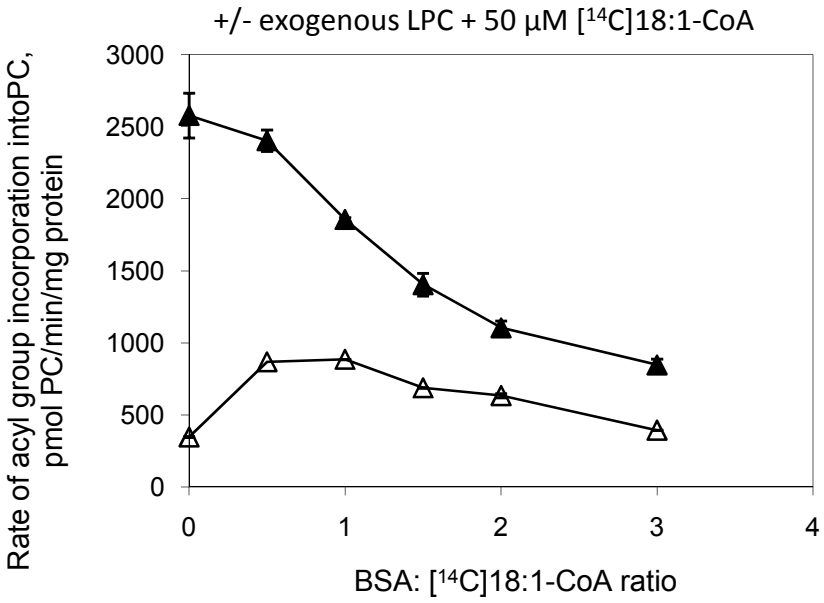


Figure 4.5 Effect of BSA on [1-^{14}C]18:1 incorporation from acyl-CoA into PC, when exogenous LPC is included (filled triangles) or omitted (open triangles) from the assay. [1-^{14}C]18:1-CoA (50 µM) and 50 µM CoA (50 µM) were incubated with microsomes (~30 µg) from ΔYOR175c yeast transformed with *AthLPCAT-1* (n=3, mean ± SE).

The amount of the radiolabel incorporated into PC from [1-^{14}C]18:1-CoA increased with increasing ACBP concentration up to the highest R tested (1.66) where incorporation was 2.5 times higher than in the absence of ACBP. BSA also stimulated incorporation up to the point of R=1, after which incorporation decreased (Figure 4.6a, 4.5). Similar results were observed when microsomes from *B. napus* MD cell suspension cultures were used (Figure 4.6b). Synthesis of the radiolabeled PC where no exogenous LPC was provided can partially be explained by acylation of the endogenous LPC. Another mechanism that could contribute to formation of radiolabeled PC is acyl exchange between acyl-CoA and endogenous PC, catalyzed by the reverse and forward reactions of LPCAT.

The effect of rBnACBP on the ability of AthLPCAT to catalyze the transfer of acyl groups from PC to the acyl-CoA was also studied. Exogenous PC with [1-^{14}C]18:2 at the *sn*-2 position was introduced to the lyophilized yeast microsomes expressing AthLPCATs, and the appearance of the radiolabel in acyl-CoA fraction was monitored. No radiolabeled acyl-CoA was formed when BSA or rBnACBP was omitted from the reaction medium, nor in assays with membranes from the yeast expressing *lacZ* (Figure 4.7). Addition of BSA to the assay resulted in a linear increase in production of the radiolabeled acyl-CoA up to the point of R=1. The recombinant BnACBP also stimulated transfer of [1-^{14}C]18:2 to acyl-CoA even beyond the point when R reached 1.

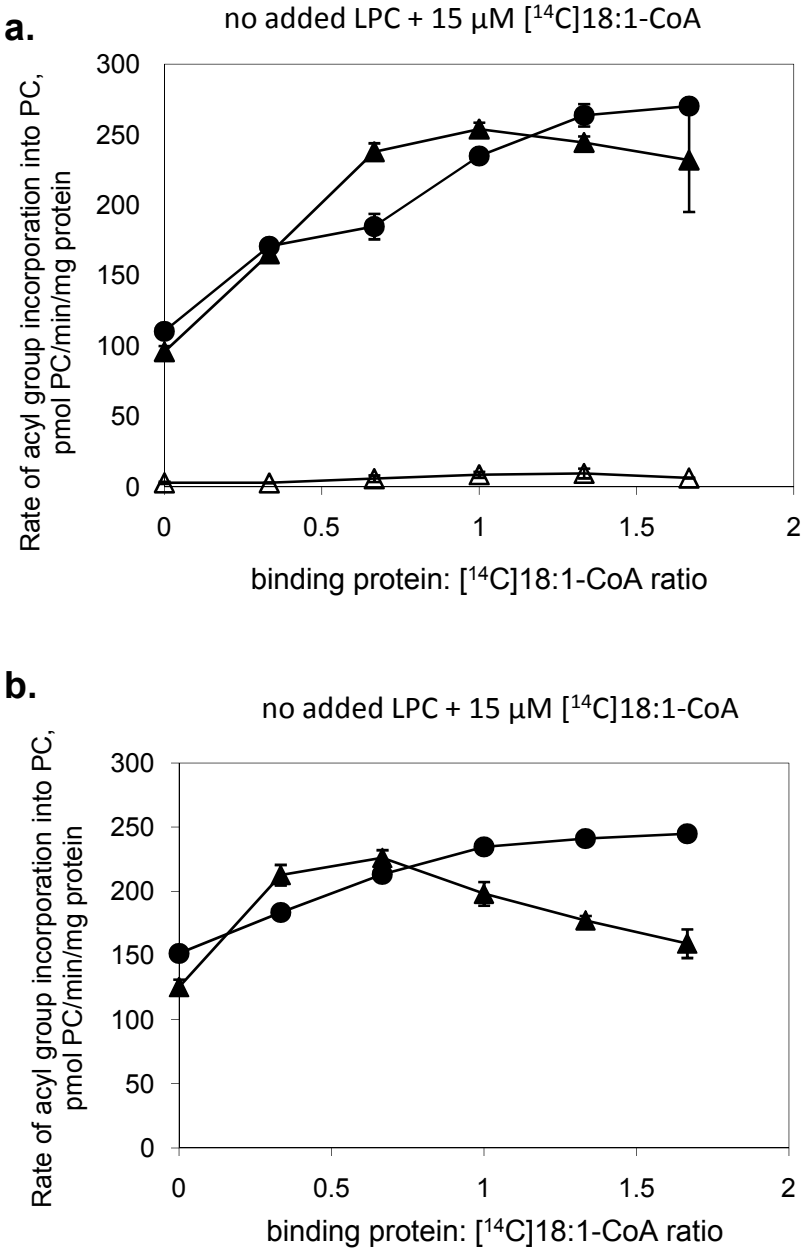


Figure 4.6 Effect of rBnACBP (circles) or BSA (triangles) on [1-^{14}C]18:1 incorporation from acyl-CoA into PC in the absence of exogenous LPC. [1-^{14}C]18:1-CoA (15 µM) and CoA (50 µM) were incubated with microsomes (~50 µg) from (a) ΔYOR175c yeast transformed with AthLPCAT-2 (filled markers) or lacZ (open markers); or (b) MD cell suspension cultures of *B. napus* L. cv Jet Neuf (n=3, mean ± SE).

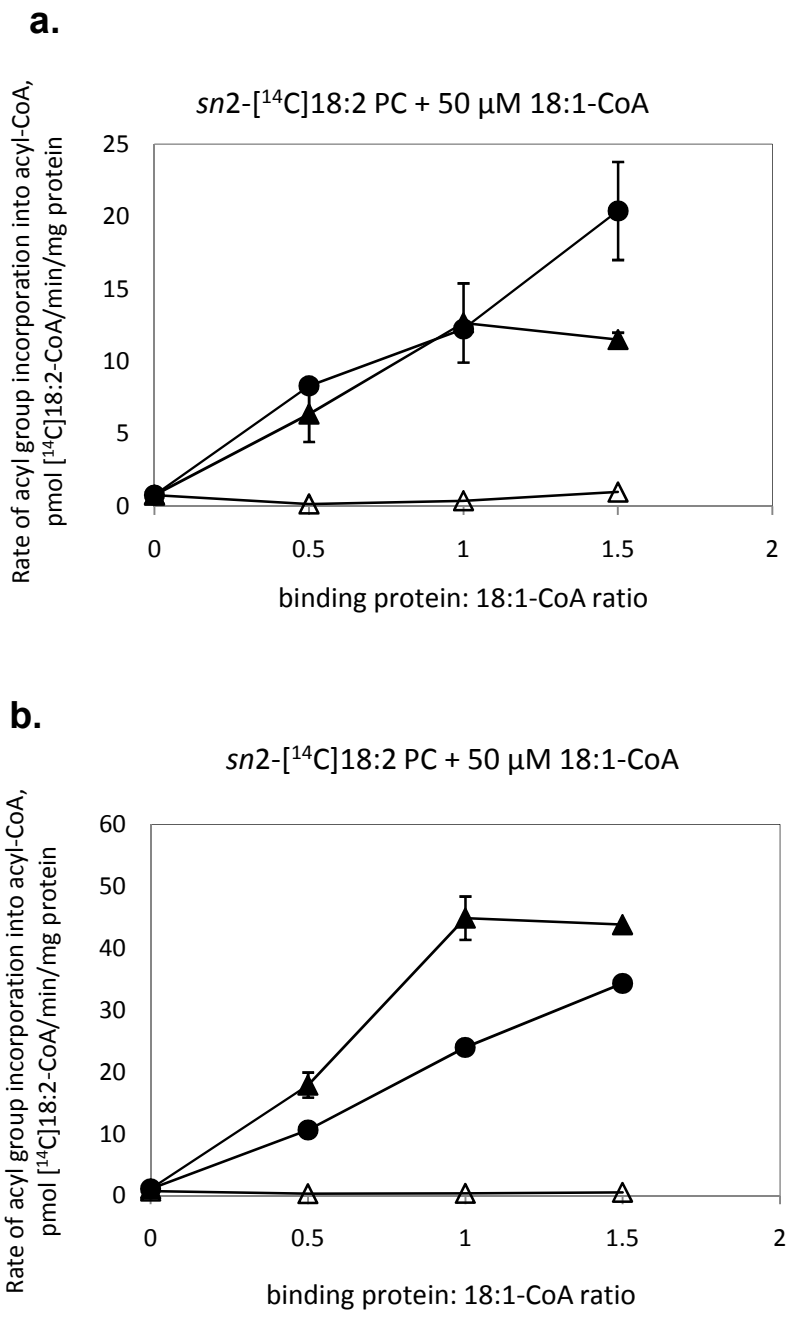


Figure 4.7 Effect of rBnACBP (circles) or BSA (triangles) on the transfer of acyl groups from PC to acyl-CoA by LPCAT (reverse reaction). *sn*-2[1-^{14}C]18:2 PC (50 µM), 18:1-CoA (50 µM) and CoA (0.2 mM) were incubated with microsomes (~15 µg) from ΔYOR175c yeast transformed with (a) *AthLPCAT-1* or (b) *AthLPCAT-2* (filled markers) or lacZ (open markers) (n=2, mean ± SE).

4.3.3 Recombinant BnACBP may compete with DGAT for acyl-CoA moieties

Yeast H1246 strain deprived of TAG synthesis was used for production of BnDGAT1 isoform. Incubation of the yeast microsomes with small amounts of rBnACBP (R=0.33) increased DGAT activity by 20%, but from that point on, further addition of rBnACBP to the reaction mixture dramatically decreased TAG formation compared to the reaction with no rBnACBP (Figure 4.8a). When DGAT activity was assayed in MD cell suspension microsomes a similar effect was found. At low R= 0.33 both rBnACBP and BSA slightly increased DGAT activity by 8% and 14% respectively (Figure 4.8b). As the concentration of BSA or rBnACBP in the assay increased, the amount of produced TAG declined and at R=1.66, DGAT activity decreased by 28% and 23%, respectively, compared to the assay with no binding protein added.

4.3.4 Effect of the recombinant BnACBP on *de novo* DAG synthesis (GPAT activity)

In microsomes of developing seeds, G3P is utilised for *de novo* synthesis of DAG by the first two acyltransferases of Kennedy pathway, GPAT and LPAAT combined with phosphatidic acid phosphatase action. We have examined the effect of rBnACBP on incorporation of [U-^{14}C]G3P into glycerolipids, mainly LPA, PA and DAG.

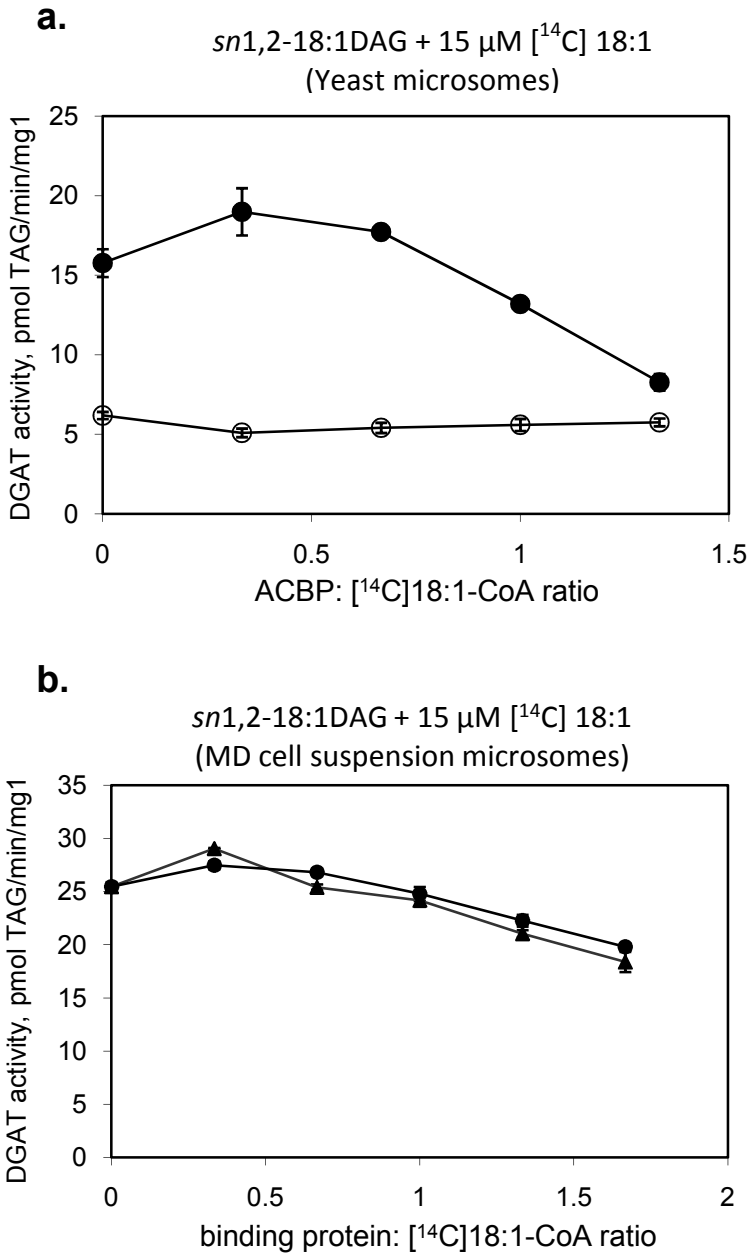


Figure 4.8 Effect of rBnACBP (circles) or BSA (triangles) on DGAT activity. [1-^{14}C]18:1-CoA (15 µM) and *sn*-1,2-diolein (330 µM) were incubated with microsomes (~50 µg) from (a) H1246 yeast expressing BnDGAT (filled markers) or lacZ (open markers) (n=3, mean ± SE) or (b) MD cell suspension cultures of *B. napus* L. cv Jet Neuf.

In membranes from MD cell suspension cultures of *B. napus,* glycerolipid synthesis from [U-^{14}C]G3P was significantly decreased at all rBnACBP concentrations tested, while BSA increased GPAT activity by around 60% at R= 0.33, after which enzyme activity started to decline, but remained above the level when no BSA was supplied (Figure 4.9a). GPAT activity was also assayed using microsomes from GPAT knock-out yeast strain expressing *B. napus* GPAT4. In contrast to the experiment described above, rBnACBP caused a linear increase in GPAT activity in yeast microsomes up to 70% at the highest R tested (1.66) (Figrure 4.9b). Addition of BSA to the reaction medium increased GPAT activity 3-fold at R=0.66 (Figure 4.9b). GPAT activity decreased with further addition of BSA and at R=1.66 it almost returned to the original level, observed at zero BSA.

4.3.5 Cloning and analysis of BnACBP variants

Using a BnACBP cDNA sequence as a query to search the *Brassica* EST database, similar sequences were identified and clustered into three groups. Primers were designed to amplify three variants of *BnACBP* from *B. napus* cDNA library. PCR products (*BnACBP-1*, *-2* and *-3*) were cloned into pCR4 vectors and sequenced. Sequences of the cloned *BnACBP* variants were compared (Figure 4.10). Amino acid substitutions in BnACBP isoforms are summarized in Table 4.1.

BnACBP1 (variant 1) had 97.8% identity to the BnACBP sequence used in this project. BnACBP1 has two substitutions in non-conserved N-terminal residues (D6E and H10Q). These amino acid substitutions resulted in a slight change of the isoelectric point from 5.41 in BnACBP to 5.19 in variant 1.

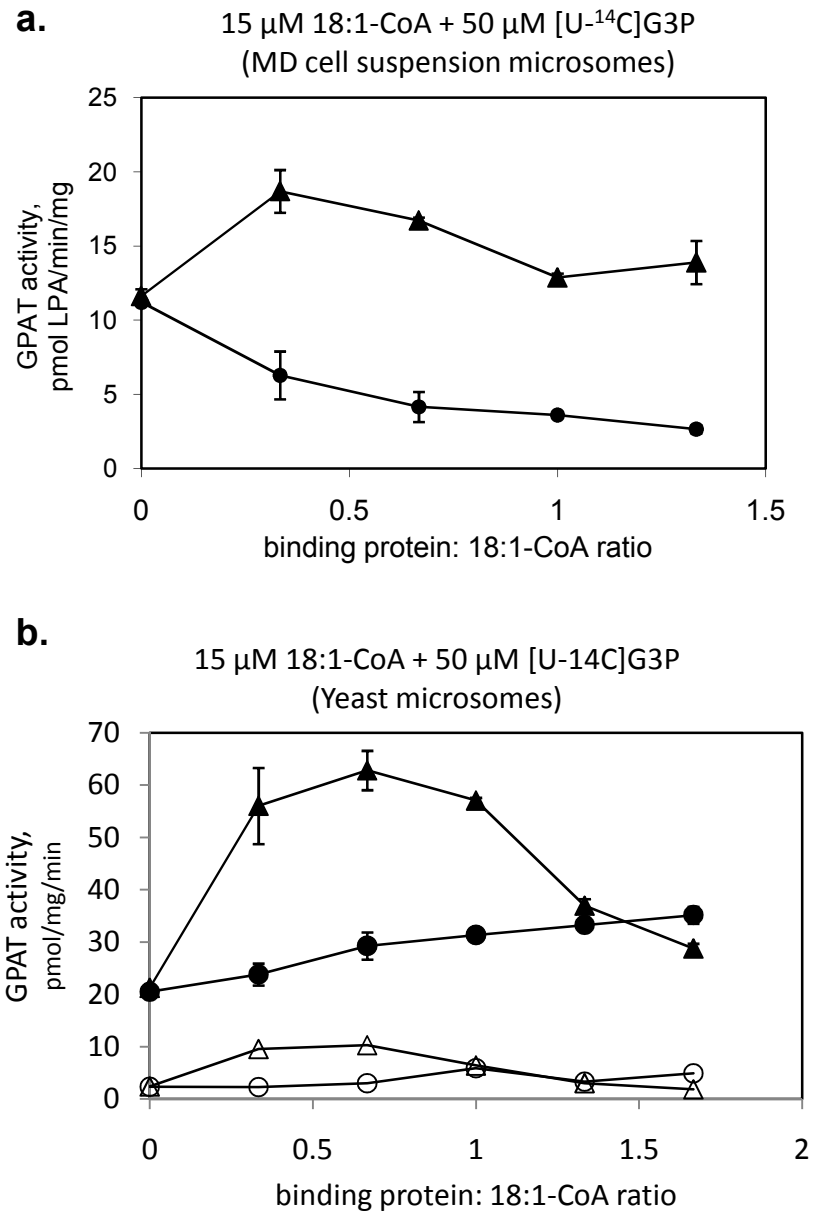


Figure 4.9 Effect of rBnACBP (circles) or BSA (triangles) on GPAT activity. 18:1-CoA (15 µM) and [U-^{14}C]G3P (50 µM) were incubated with microsomes (~50 µg) from (a) MD cell suspension cultures of *B. napus* L. cv Jet Neuf or (b) yeast transformed with *BnGPAT4* (filled markers) or *LacZ* (open markers) (n=3, mean ± SE).

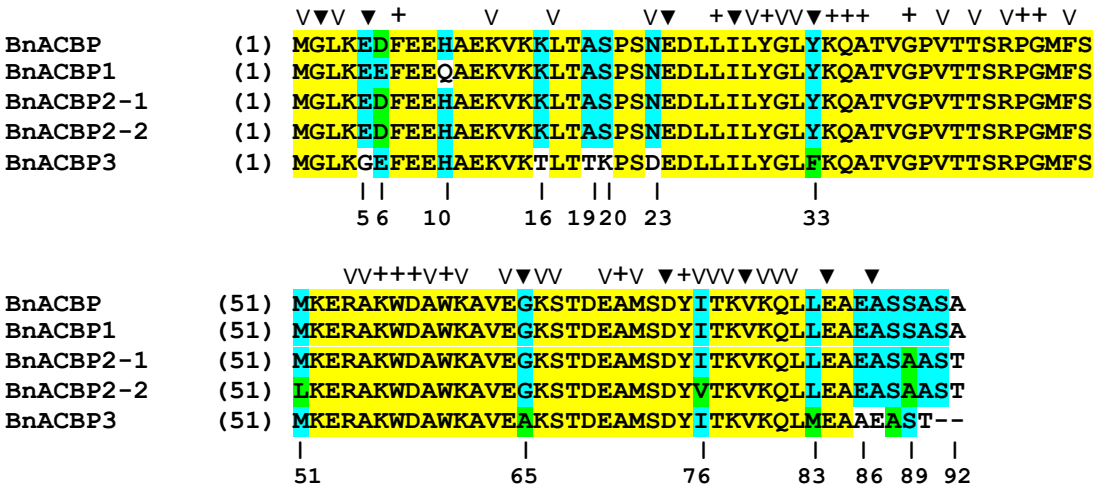


Figure 4.10 Sequence alignment of BnACBP (X77134) with BnACBP variants cloned from *B. napus* cDNA. A plus sign (+) indicates amino acids conserved in all ACBPs; an open arrow (V) indicates amino acid residue conserved in plant ACBPs; a filled arrow (▼) represents amino acid residues proposed to be conserved in plant ACBPs by Engeseth *et al.* (1996), but displaying a certain degree of variation in plant ACBPs. Numbers under the sequence alignment indicate positions of the amino acid substitutions compared to the query BnACBP (X77134).

Table 4.1. Amino acid (aa) substitutions in BnACBP variants, compared to BnACBP (X77134). Bold italicized pairs represent substitutions of amino acids considered to be conserved in plants.

ACBP variant	aa substitution	substituted aa	aa substituted for
BnACBP1	D6E	Asp (polar-)	Glu (polar-)
	H10Q	His (polar+)	Gln (polar)
BnACBP2-1	S89A	Ser (polar)	Ala (nonpolar)
	A92T	Ala (nonpolar)	Thr (polar)
only in BnACBP2-2	M51L	Met (nonpolar)	Leu (nonpolar)
	I76V	Ile (nonpolar)	Val (nonpolar)
BnACBP3	***E5G***	Glu (polar-)	Gly (nonpolar)
	D6E	Asp (polar-)	Glu (polar-)
	K16T	Lys (polar+)	Thr (polar)
	A19T	Ala (nonpolar)	Thr (polar)
	S20K	Ser (polar)	Lys (polar+)
	N23D	Asn (polar)	Asp (polar-)
	Y33F	Tyr (polar)	Phe (nonpolar)
	G65A	Gly (nonpolar)	Ala (nonpolar)
	L83M	Leu (nonpolar)	Met (nonpolar)
	E86A	Glu (polar-)	Ala (nonpolar)

BnACBP2 (variant 2) was represented by two very similar sequences. The first sequence (BnACBP2-1) is 97.8% identical to BnACBP, and differs only in two amino acids at the C-terminus (S89A and A92T). Both substituted amino acids are not conserved in plant ACBPs and do not affect the net charge of the protein. The second sequence (BnACBP2-2) has two additional amino acid substitutions (M51L and I76V). Leu at position 51 is also found in amino acid sequences of small cytosolic ACBPs from *Oryza sativa* and *Zea mays* (Figure 2.3). Ile at the position 76 is considered to be conserved in plants (Engeseth *et al.*, 1996). This substitution, however, does not change the charge of the molecule.

BnACBP3 (variant 3) is the most divergent variant and shares 83.7% identity with BnACBP sequence. Variant 3 has 10 substitutions and is 2 amino acids shorter than BnACBP. Amino acid substitutions of E5G, N23D, Y33F and G65A are considered to be conserved in plants according to Engeseth *et al.* (1996), are found in ACBP sequences from other species. Phe-33 and Ala-65 are present in ACBP from *Linum usitatissimum*, and Asp at the position 23 is found in animal ACBP sequences (Figure 2.3). The last three amino acids of BnACBP3, AST, are the same as in variant 2, but the preceding four amino acids (EASA) are shortened to AE in variant 3. With all the amino acid substitutions, the net charge of the molecule should remain unchanged. Amino acids that constitute the binding domain and those that are conserved in all species remain unchanged in the BnACBP variants. Whether these changes in amino acid sequence affect the protein conformation and/or binding properties remains to be determined.

4.4 Discussion

Small soluble ACBPs have been shown previously to affect enzymatic activities that have acyl-CoA moieties as a substrate or a product (Brown *et al.*, 1998; Brown *et al.*, 2002; Fyrst *et al.*, 1995; Jolly *et al.*, 2000; Kannan *et al.*, 2003; Huang *et al.*, 2005). In our *in vitro* studies we evaluated the effect of rBnACBP on acyltransferases involved in seed oil biosynthesis.

Results of *in vitro* LPCAT assays suggested that ACBP may enhance incorporation of 18:1 into PC by promoting the acyl exchange between PC and acyl-CoA catalyzed by LPCAT. An increased rate of 18:1 esterification to PC creates more substrate for FAD2 desaturase, which could result in higher production of 18:2, which can get desaturated further to 18:3 by FAD3. FAD2 and FAD3 desaturases are known to catalyze the formation of 18:2 and α-18:3 in PC, respectively (Ohlrogge and Browse, 1995). Whether PUFAs produced on PC return to the cytosolic acyl-CoA pool via acyl editing or get channelled to seed oil though acyl-CoA independent pathways remains unknown. Based on the results of our *in vitro* assays and analysis of the acyl-CoA pool of *A. thaliana* seeds overexpressing BnACBP, we propose that 10 kDa ACBP enhances acyl editing by the removal of PUFA from PC, thus, enriching the acyl-CoA pool with PUFA and producing endogenous LPC that can be re-acylated with *de novo* synthesized 18:1. BSA or rBnACBP had a similar stimulatory effect on the reverse reaction of LPCAT and incorporation of [^{14}C]18:1-CoA into endogenous PC, while these binding proteins had an opposite effect on the forward LPCAT reaction, when exogenous LPC was provided. On one hand, this can mean that not only binding

of the ligand, but some additional factors such as protein-protein interactions between ACBP and acyltransferases are important. On the other hand, the fact that BSA stimulated LPCAT activity only in one direction (reverse), while ACBP increased LPCAT activity in both directions supports the notion that ACBP may be an important component of the acyl editing system. Effects of BSA on the forward and reverse reactions of LPCAT also agree with the previously published data (Stymne and Stobart, 1984; Furukawa-Stoffer *et al.*, 2003).

Stymne and Stobart (1984) proposed that BSA stimulation of the LPCAT reverse reaction can be explained by the change in free acyl-CoA: CoA ratio in the cytosol, which could shift the LPCAT reaction towards the removal of acyl groups from PC. BSA, however, is a mammalian protein. ACBP is the likely candidate to play a role in LPCAT dynamics in the plant. Recently it was demonstrated that recombinant *A. thaliana* cytosolic 10 kDa could bind PC *in vitro* (Chen *et al.*, 2008). Whether this property remains *in vivo* and whether it has anything to do with the ACBP effect on LPCAT activity remains to be determined.

Results of the DGAT assays from yeast and MD cell microsomes suggest that ACBP may modulate DGAT utilization of acyl-CoAs. rBnACBP can slightly increase DGAT activity at acyl-CoA concentrations higher than that of ACBP (ACBP: acyl-CoA ratio<0.5). In a biological context, this observation could mean that in cases when ACBP is not able to accommodate the excess of free acyl-CoAs, DAG acylation is stimulated. At ACBP: acyl-CoA ratio≈1, which is the most reflective of the *in planta* situation, rBnACBP had little effect on DGAT

activity. But as the ACBP: acyl-CoA ratio exceeds 1, channelling of acyl groups to the *sn*-3 position of TAG by DGAT is inhibited. BSA, similarly to rBnACBP, had a limited stimulatory effect on DGAT, which somewhat differs from the results reported for *in vitro* assays from the embryo cultures of *B. napus*, where DGAT activity was stimulated 4-5-fold by BSA (Little *et al.*, 1994).

We have obtained conflicting results on the effect of rBnACBP on GPAT activity, when using microsomes from MD cell suspension culture and microsomes from yeast producing BnGPAT. This may be partially explained by the difference in the membrane composition in the two systems, including surrounding phospholipids and enzymes, which can affect substrate availability as well as product channelling. It appears that the GPAT product LPA does not accumulate in microsomes, but instead gets rapidly used by LPAAT producing PA, which can be then converted to DAG. Therefore, GPAT activity was quantified based on total radioactivity in LPA, PA and DAG. Using a yeast knock-out system, where the only GPAT activity comes from the transgenic BnGPAT, we observed an increase in GPAT activity in response to increasing concentrations of rBnACBP, even beyond ACBP: acyl-CoA ratio of 1. On the other hand, BSA caused an increase in GPAT activity in both microsome systems in a similar manner. Production of glycerolipids from G3P tripled in yeast microsomes and increased 60% in MD cell suspension microsomes at BSA: 18:1-CoA ratio<1, but at the ratio>1 GPAT activity decreased to the original level. These results are similar to those of rBnACBP on GPAT in *B. napus* embryos (Brown *et al.*, 1998).

Based on the *in vitro* studies of rBnACBP effects on LPCAT, DGAT and GPAT, we conclude that the most prominent effect of small cytosolic ACBP in seed oil biosynthesis is on acyl exchange between acyl-CoA and PC catalyzed by LPCAT. The effect of ACBP on G3P utilization for the *de novo* synthesis of DAG depends on the physiological characteristics of the system. DGAT activity is modulated by rBnACBP when ACBP: acyl-CoA ratio deviates from physiologically relevant levels.

It is likely that the *in vivo* effect of ACBP on the enzymes of seed oil biosynthesis will depend not only on the ACBP concentration (or ACBP: acyl-CoA ratio) in the tissue, but also the composition of different ACBP isoforms and binding specificities of those. It has been shown previously that the *B. napus* genome contains at least six ACBP genes (Hills *et al.*, 1994). This is not surprising considering that the ACBP family of six genes has been already characterized in *A. thaliana* (Reviewed in Xiao and Chye, 2009). Multiple ACBP isoforms that differ in isoelectric point and expression pattern have also been described in *B. napus* (Brown *et al.*, 1998). Whether those isoforms represent different members of ACBP family or if they are variants of the same ACBP, remains unclear. We have identified and cloned three cDNAs encoding three variants of the small soluble 10 kDa ACBP from *B. napus*. Amino acid sequences of these isoforms were 83.7-97.8% identical to the sequence of *B. napus* ACBP originally published (Hills *et al.*, 1994). The temporal production of these isoforms during seed development and their binding properties remain to be studied.

5. Suppression of *ACBP* expression in *A. thaliana*.

5.1 Introduction

Reverse genetics, in which a gene is disrupted so that the effect (if any) of its loss on an organism can be observed, is a simple way to investigate gene function (Reviewed in Alonso and Ecker, 2006). A reverse-genetic approach called insertional mutagenesis has been a key in studying gene function in *Arabidopsis*. Insertional mutagenesis is based on disruption of gene expression through the random insertion of foreign DNA (T-DNA or transposon) into the genome (Krysan *et al.*, 1999). The foreign DNA also serves as a marker for identification of the presence and position of the mutation. The use of T-DNA insertion is more common than the use of transposons, since it results in a mutation stable through multiple generations. T-DNA that is integrated within the boundaries of a gene usually causes a complete abolishment of the gene function (a gene knock-out). Large-scale application of the insertional mutagenesis and cataloguing of the locations of the T-DNA inserts in the mutagenized populations have given rise to several large, publicly available collections of *Arabidopsis* insertion mutants, such as the SALK collection in The Arabidopsis Information Resource (TAIR) and the European *Arabidopsis* Stock Centre.

The first knock-out mutant for the gene encoding ACBP in Arabidopsis was described for a membrane bound member of the ACBP family (Mishra *et al.*, 2004). Analysis of the total lipids from the mature green leaves of the *acbp2* mutant (CS19943, Thomas Jack pool) showed a significant increase in 16:0 at the

expense of α-18:3. Plants also displayed lateral root inhibition, and showed a short bolt phenotype and gibberellic acid (GA)-insensitivity at cold temperature (10^{o}C). These results pointed out the possibility of ACBP2 involvement in auxin signalling and in GA signalling under cold stress. T-DNA knock-out lines have also been identified and characterized for two cytosolic proteins ACBP4 (SALK_040164) and ACBP6 (SALK_104339) (Xiao *et al.*, 2008b; Chen *et al.*, 2008). The *acbp4* mutant showed decreases in membrane lipids (digalactosyldiacylglycerol, monogalactosyldiacylglycerol, phosphatidylcholine, phophatidylethanolamine and phosphatidylinositol) in the leaves, suggesting that ACBP4 plays a role in the biosynthesis of membrane lipids including galactolipids and phospholipids (Xiao *et al.*, 2008b). The *acbp6* T-DNA mutant exhibited enhanced sensitivity to freezing stress, which was associated with the phospholipase Dδ-mediated pathway (Chen *et al.*, 2008).

Although T-DNA insertion mutants have proven to be a great resource, there are some limitations to the use of this technology. Due to the random nature of T-DNA insertion, the desired mutations in some genes of interested might not be found. If the T-DNA insert has been identified in the gene of interest, several rounds of backcrossing of the mutant line to the parental wild-type (WT) lines might be required due to the possibility of multiple T-DNA insertions in the genome. Some knock-out mutations are lethal, and in that case the T-DNA line can not be maintained or analyzed. Insertional mutagenesis is also not very suitable for studying tandemly repeated genes or gene families with closely related members.

One approach that can circumvent some of these limitations is RNA-induced gene silencing, also called RNA interference (RNAi) (Reviewed in Vance and Vaucheret, 2001; Waterhouse and Helliwell, 2003). In this technology, introduction of double-stranded RNA triggers a sequence-specific RNA degradation mechanism that effectively silences a targeted gene. When a plant is transformed with a construct encoding a double-stranded RNA (hairpin RNA) and the construct is expressed, a silencing signal is generated that can spread throughout the plant causing transcript degradation of the endogenous gene of interest. Unlike knock-out mutants, RNAi lines typically show a wide range of effects on gene expression, from complete inhibition to no reduction. The RNAi approach also offers an opportunity to silence gene expression at specific developmental stages or in specific tissues. The RNAi construct is genetically dominant and therefore phenotypes can be screened in T_0 or T_1 plants without the need to produce homozygous lines. Development of new vectors for cloning and delivery of the hairpin RNA-encoding constructs has enhanced the use of RNAi technology for gene silencing in plants (Helliwell and Waterhouse, 2003). *A. thaliana* RNAi lines produced for silencing of the *ACBP2* gene showed, similarly to *acbp2* T-DNA mutant, a significant decrease in the unsaturation level of the acyl groups of phospholipids in rosette leaves compared to WT (Kojima *et al.*, 2007).

Silencing of gene expression can also be accomplished by introducing an antisense transgene (Ecker and Davis, 1986; Vance and Vaucheret, 2001). Whether antisense transgene RNA triggers degradation of homologous

endogenous RNA in a manner similar to RNAi or has a specific mechanism, is unclear. The use of this technology is not very common due to the weak silencing by the single-copy antisense transgenes. Antisense technology was used for studying the effect of the small cytosolic ACBP on FA composition of seed oil in *B. napus* cv. Tantal, high in erucic acid (Enikeev and Mishutina, 2005). Transformation with antisense cDNA of *A. thaliana* ACBP resulted in 1.5 – 2-fold increase in erucic acid content as compared to control.

In the current study, RNAi technology was used for *AthACBP* (At1g31812) gene silencing in *A. thaliana* by expressing hairpin RNA constructs constitutively or in developing seeds. Our primary interest was to investigate the effect of the *AthACBP* suppression on FA composition of seed oil.

5.2 Experimental Procedures

5.2.1 Cloning of *AthACBP_RNAi* constructs

Two binary vectors pSBS4148 and pSBS4149 with *AthACBP-RNAi* constructs for constitutive expression were assembled through Gateway cloning of *AthACBP* into pWatergate and pHellsgate12 (CSIRO Plant Industry, Townsville, Australia), respectively (Figure 5.1). Complete description of the cloning procedures for making *AthACBP* hairpin constructs is presented in Appendix 1.

AthACBP_RNAi construct for seed specific gene silencing was assembled on pHannibal cloning vector (CSIRO Plant Industry, Townsville, Australia) using conventional cloning methods. *AthACBP_RNAi* cassette was then cloned into pGreen0229 binary vector (based on pGreen0029, John Innes Centre, Norwich, UK), resulting in pGreen-AthACBP_RNAi plasmid (Figure 5.2, A1.5).

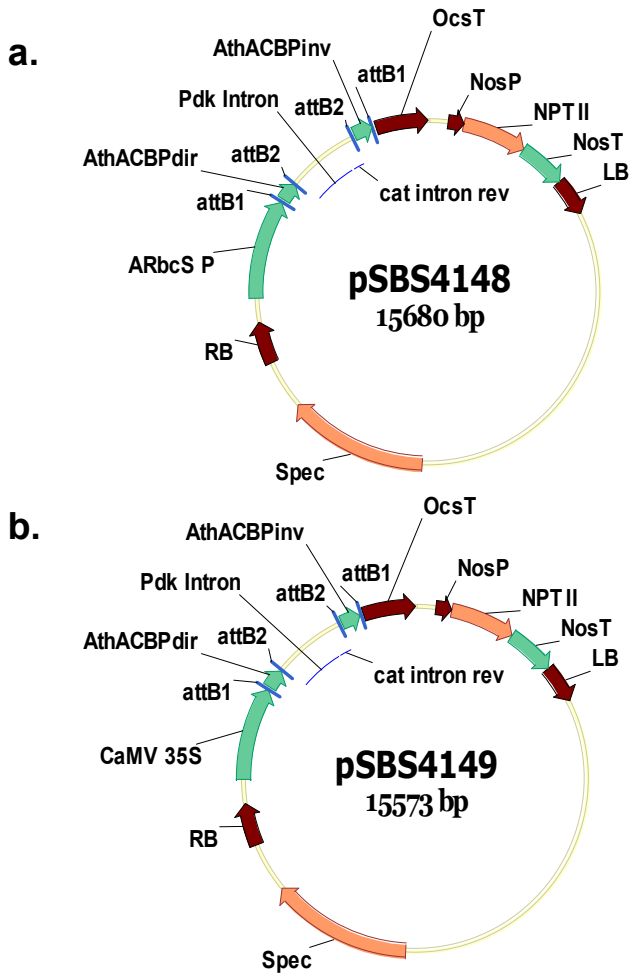


Figure 5.1. Molecular maps of the binary vectors used for *A. thaliana* transformation with *AthACBP_RNAi*(cons) constructs. a. pSBS4148; b. pSBS4149. Components of the T-DNA constructs: AthACBPdir and AthACBPinv – *A. thaliana* ACBP cDNA direct and inverted sequences, respectively; ARbcS P – *Arabidopsis* rubisco small subunit promoter; CaMV 35S – cauliflower mosaic virus 35S promoter; Pdk Intron – pyruvate orthophosphate dikinase intron (from *Flaveria trinervia)*; cat intron rev – catalase intron; OcsT – octopine synthase terminator; attB1 and attB2 – recombination sites; NosP and NosT- nopaline synthase promoter and terminator, respectively; NPTII – neomycin phosphotransferase II gene (selection marker responsible for the kanamycin tolerance of the transgenic plants); RB and LB – left and right borders of T-DNA.

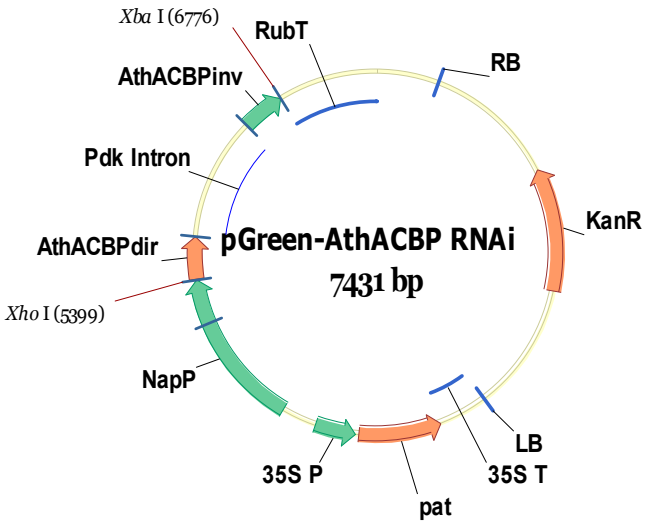


Figure 5.2. Molecular map of the binary vector used for *A. thaliana* transformation with *AthACBP_RNAi*(Nap) construct. Components of the T-DNA constructs: AthACBPdir and AthACBPinv – *A. thaliana* cytosolic ACBP cDNA direct and inverted sequences, respectively; NapP – napin promoter; RubT – rubisco terminator; Pdk Intron – pyruvate orthophosphate dikinase intron (from *Flaveria trinervia)*; 35S P and 35S T - cauliflower mosaic virus 35S promoter and terminator, respectively; pat - phosphinotricin acetyltransferase gene (selection marker responsible for the phosphinotricin tolerance of the transgenic plants); RB and LB – left and right borders of T-DNA.

pSBS4148 and pSBS4149 were used for *Agrobacterium* EHA101 transformation. pGreen-AthACBP_RNAi was introduced into GV3101 *Agrobacterium* strain with the addition of pSoup plasmid. *A. thaliana* transformation and growth conditions were the same as described in Chapter 3.

5.2.2 Analysis of *AthACBP* expression by qRT-PCR

Total RNA was extracted from *A. thaliana* young leaves and developing siliques using RNeasy Plant MiniKit (Qiagen, Mississauga, ON, Canada) and Trizol reagent (Invitrogen, Burlington, ON, Canada), respectively, following the manufacturers' protocols. RNA extraction from the developing seeds was performed following the protocol of Herzog (1995) with slight modifications. Developing seeds (15 DAF) were frozen in liquid nitrogen and homogenized in a bead beater (BioSpec Products Inc.) for 1 min with a 3 mm Zirconia/Silica bead (BioSpec Products Inc., Bartlesville, OK, USA). 450 µL Buffer (1M Tris pH 9.0, 1% SDS), 300 µL Phenol: Chloroform: Isoamyl alcohol (25:24:1) saturated with 10mM Tris, pH8.0, 1mM EDTA, 75 µl 10% PVP-40, and 6 µL β-mercaptoethanol were added to the tissue and the sample was vortexed for 20 min at room temperature. After centrifugation of the sample at 2000 ***g*** at 4°C for 20 min, the aqueous phase was transferred to a clean tube and combined with two volumes of the ethanol and 1/10 volume of 3M sodium acetate. The mixture was incubated at -20°C for 1 h and centrifuged at 12,000 x ***g*** for 20 min at 4°C. The resulting pellet was resuspended in 250 µL of 2M LiCl and incubated on ice overnight. The mixture was then centrifuged at 6000 ***g*** for 20 min at 4°C. The pellet was washed once with 2M LiCl and resuspended in 25 µL of RNAse-free

water. Concentration and purity of RNA samples were assessed by spectrophotometry and agarose gel electrophoresis, respectively.

Synthesis of cDNA was performed using Quantitech Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada). Expression levels of *AthACBP* were measured by qRT-PCR using TaqMan Gene Expression Assay At02169508 (Applied Biosystems, Streetsville, ON, Canada). Protein phosphatase 2A subunit gene (*PP2A*, At1g13320) was used as an endogenous control. TaqMan MGB Probe for PP2A with VIC-dye (PP-VIC) was custom made by Applied Biosystems and used with PP-F and PP-R primers (Table A1.1). qRT-PCR was performed on 7900HT Fast Real-Time PCR System with SDS software (Applied Biosystems, Streetsville, ON, Canada).

Ct (threshold cycle) values generated by the SDS software for the target gene (*AthACBP*, At1g31812) and endogenous control (*PP2A*, At1g13320) were used to calculate the RQ (relative quantity) value for each test sample (RNAi), which is a fold change of the sample compared to the calibrator sample (WT). Ct value represents the PCR cycle number at which the fluorescence passes the threshold level set in the exponential growth region of the amplification curve. First, the difference between the Ct of the target signal and the Ct of the corresponding endogenous reference for each sample is calculated [ΔCt = Ct (target) – Ct (endogenous control)]. Then, the difference between the average ΔCt value of a test sample and the average ΔCt for the calibrator sample is calculated [$\Delta\Delta$Ct = ΔCt (test sample) - ΔCt (calibrator sample)]. Finally, the RQ for each test sample was calculated used the formular $RQ = 2^{-\Delta\Delta Ct}$.

5.2.3 Biochemical analysis of seed oil

Direct transmethylation of 10 mg of mature T_2 seeds with methanolic HCl was used for seed oil analysis of *AthACBP_RNAi* lines. FAME samples in iso-octane were analyzed on Agilent 6890N Gas Chromatograph with 5975 inert XL Mass Selective Detector equipped with an autosampler. FAMEs were separated using a capillary DB-23 (30m) column (0.25 mm x 0.25 µm x 30 m) with constant He flow 1.2 mL/min and a temperature program: 90-180°C at 10°C/min, hold at 180 for 5 min, 180-230°C at 5°C/min. Integration events were detected and identified between 9 and 20 min. Statistical analysis of the data was performed as described in Chapter 3.

5.3 Results

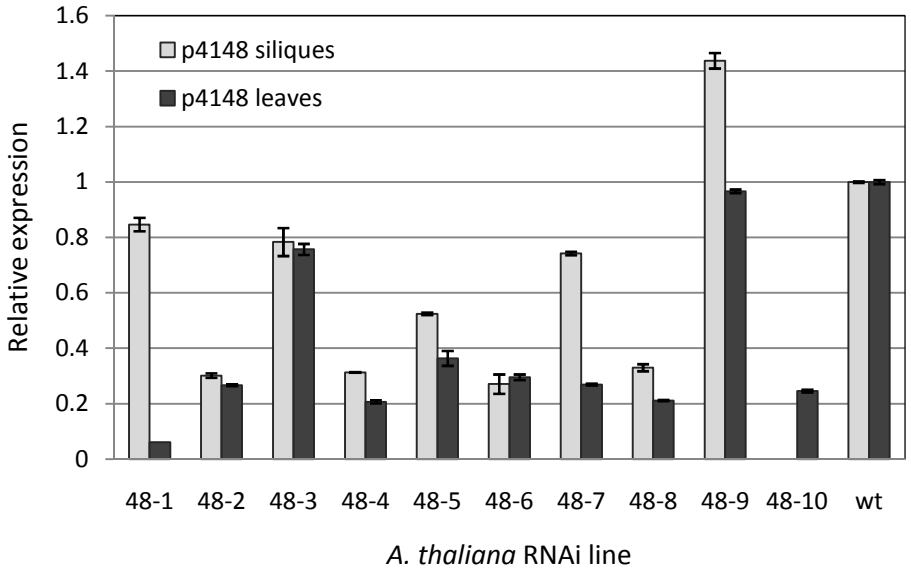
5.3.1 Analysis of *AthACBP* transcript levels in *A. thaliana* RNAi lines

Two binary vectors pSBS4148 and pSBS4149 vectors were made for *AthACBP* gene silencing through constitutive expression of RNAi constructs, *AthACBP_RNAi*(cons). *Agrobacterium*-mediated transformation of *A. thaliana* with these vectors resulted in 10 lines for each construct. Quantitative RT-PCR (qRT-PCR) was performed in order to evaluate levels of *AthACBP* suppression by *AthACBP_RNAi*(cons) constructs. Total RNA was extracted from the green leaves and developing siliques collected from *A. thaliana* T_1 plants transformed with pSBS4148 and pSBS4149 harbouring *AthACPB_RNAi* constructs for constitutive expression. After the synthesis of cDNA, real-time RCR was performed with TaqMan Gene Expression Assay specific for *AthACBP* gene (At1g31812). Separate real-time PCR reactions were set with the probe and primers specific for

protein phosphatase 2A subunit gene (*PP2A*, At1g13320), which was used as an endogenous control for normalization of the amount of template. This gene has been shown to have a superiror expression stability compared to the tranditional reference genes in *Arabidopsis* (Czechowski *et al*., 2005). Each reaction, for *AthACBP* and *PP2A*, was done in triplicate. Most of the analyzed *AthACBP_RNAi*(cons) lines displayed some degree of *AthACBP* silencing. Four out of 10 lines transformed with pSBS4148 had 27-32% of the WT *AthACBP* expression levels in developing siliques (Figure 5.3a). *AthACBP* transcript levels in siliques of the other lines transformed with pSBS4148 were more than a half of the WT levels.

Similar results were observed in *A. thaliana* line transformed with pSBS4149 (Figure 5.3b). Five out of 10 lines transformed with pSBS4149 displayed 21-31% of the WT levels of *AthACBP* expression in developing siliques. The rest of the pSBS4149 lines had at least a half of the normal *AthACBP* transcript levels. We also observed consistently higher levels of *AthACBP* suppression in leaf tissue than in developing siliques for both *AthACBP_RNAi*(cons) constructs (Figure 5.3). This can be at least partially explained by activity of the constitutive promoters (CaMV 35S and ARbcS P) that can vary depending on the plant tissue and plant species transformed (Schaffner and Sheen, 1991; Wilkinson *et al.*, 1997). In order to increase the level of *AthACBP* suppression in developing seed we made an *AthACBP_RNAi* construct expressed under the seed specific napin promoter, *AthACBP_RNAi*(Nap).

a.



b.

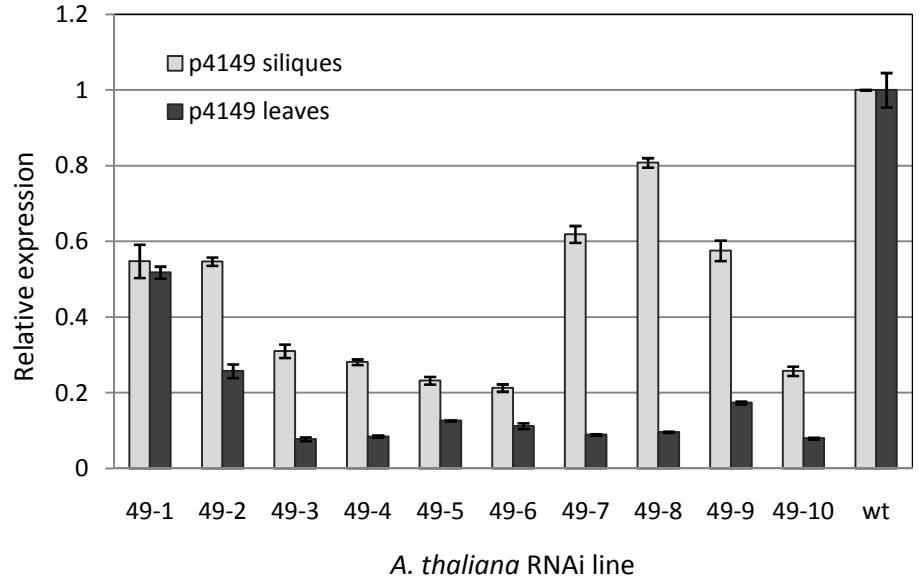


Figure 5.3. Relative levels of *AthACBP* expression in developing siliques (16 DAF) (light colored bars) and green leaves (dark colored bars) from *A. thaliana* T_1 lines transformed with *AthACBP_RNAi*(cons) constructs a. pSBS4148, and b. pSBS4149, compared to WT plants (RQ ± SD, n=3). *AthACBP* expression levels were normalized by *PP2A* levels. (Data point for the silique sample of 48-10 line is not available).

A. thaliana plants were transformed with the binary vector pGreen-AthACBP_RNAi harbouring *AthACBP_RNAi*(Nap) construct, giving rise to 12 independent transgenic lines (pGreen lines). Total RNA was extracted from developing seeds (15 DAF) from six siliques for five pGreen lines and WT. qRT-PCR was performed for pGreen1 - pGreen5 transgenic lines representative of the phenotypes observed in T_2 seed oil. All analyzed pGreen lines showed a significant decrease in levels of *AthACBP* transcript in developing seeds (Figure 5.4). *AthACBP* transcript level in pGreen1 line, which displayed significant changes in FA composition of seed oil compared to WT, was about a half of the levels observed in WT. Other pGreen lines, which had no significant changes in FA composition in seed oil, also showed a 30-70% decrease in *AthACBP* expression compared to WT. All pGreen lines analyzed had substantial down-regulation of *AthACBP* expression, but only pGreen1 line showed significantly different FA profile of seed oil compared to WT. The positional effect of T-DNA insertion in the plant genome might be the cause of the modifications in seed oil biosynthesis in pGreen1 line.

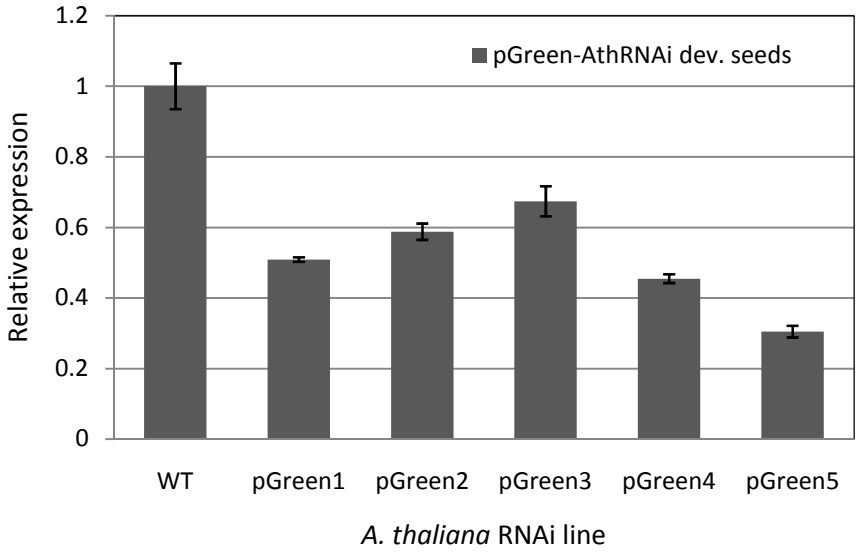


Figure 5.4. Relative levels of *AthACBP* expression in developing siliques (15 DAF) from *A. thaliana* T_1 lines transformed with *AthACBP_RNAi*(Nap) construct (pGreen-AthACBP_RNAi), compared to WT plants (RQ ± SD, n=3). *AthACBP* expression levels were normalized by *PP2A* levels.

5.3.2 Analysis of the fatty acid composition of seed oil from *A. thaliana* T_2 seeds expressing *AthACBP_RNAi* constructs.

Mature heterozygous T_2 seeds were harvested from 10 lines transformed with pSBS4148 and pSBS4149, each, and analyzed in triplicates for each line. All individual lines within each construct had similar levels of variation, and were used as biological replicates of the construct. The main change in FA composition of seed oil, similar for both *AthACBP_RNAi*(cons) constructs, was an increase in 18:1 levels from 15.4% in WT to 19% in T_2 (Table 5.1). Other modifications included an increase in 18:2 and a decrease in 18:0 and α-18:3 percentages compared to WT. 20:1 levels were not altered in pSBS4149 lines, and showed small but significant decrease in seeds transformed with pSBS4148. Overall, the proportion of monounsaturated fatty acids (MUFA) in seed oil of *AthACBP_RNAi*(cons) T_2 seeds increased significantly, while SFA levels decreased compared to WT (Table 5.2). Changes in polyunsaturated fatty acids (PUFA) content were inconsistent: pSBS4148 lines had slightly elevated PUFA, while pSBS4149 lines had decreased portion of PUFA in seed oil. Twelve T_2 lines transformed with pGreen-AthACBP_RNAi contruct for seed specific suppression of *AthACBP* expression were analyzed. Eleven lines (pGreen2-12) showed no significant changes in FA composition of seed oil compared to WT (Table 5.3).

Table 5.1. FA composition of the seed oil from *A. thaliana* T_2 seeds transformed with *AthACBP_RNAi*(cons) constructs. Mean %weight ± SD of the means (n=10). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05.

Construct	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:1**
WT	7.47 ± 0.27	3.51 ± 0.19	15.42 ± 0.55	26.99 ± 0.28	16.88 ± 0.56	20.99 ± 0.17
pSBS4148	7.75 ± 0.26	2.41 ± 0.24$^{\blacktriangledown}$	19.04 ± 1.13$^{\blacktriangle}$	30.34 ± 0.89$^{\blacktriangle}$	14.79 ± 0.67$^{\blacktriangledown}$	19.21 ± 0.97$^{\blacktriangledown}$
pSBS4149	7.27 ± 0.23	2.57 ± 0.23$^{\blacktriangledown}$	19.16 ± 0.58$^{\blacktriangle}$	28.86 ± 0.47$^{\blacktriangle}$	14.23 ± 0.44$^{\blacktriangledown}$	20.68 ± 0.64

Table 5.2. Composition of FA classes in *A. thaliana* T_2 seeds transformed with *AthACBP_RNAi*(cons) constructs. Mean %weight ± SD of the means (n=10). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}$) value significantly greater than WT at α=0.1.

Construct	SFA	MUFA	PUFA
WT	13.35 ± 0.36	41.21 ± 0.37	45.43 ± 0.34
pSBS4148	11.56 ± 0.52$^{\blacktriangledown}$	42.17 ± 0.79$^{\blacktriangle}$	46.26 ± 0.99$^{\Delta}$
pSBS4149	12.54 ± 0.45$^{\blacktriangledown}$	44.21 ± 0.90$^{\blacktriangle}$	44.32 ± 0.73$^{\blacktriangledown}$

Table 5.3. FA composition of the seed oil from *A. thaliana* T_2 seeds transformed with *AthACBP-RNAi*(Nap) construct. %weight ± SD of the means. ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05.

Construct	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:0**	**20:1**
WT	7.52 ± 0.05	3.33 ± 0.03	16.57 ± 0.40	27.19 ± 0.16	15.67 ± 0.23	2.37 ± 0.03	20.63 ± 0.21
pGreen1	7.55 ± 0.03	4.36 ± 0.03$^{\blacktriangle}$	14.79 ± 0.01$^{\blacktriangledown}$	23.41 ± 0.09$^{\blacktriangledown}$	17.74 ± 0.02$^{\blacktriangle}$	3.41 ± 0.02$^{\blacktriangle}$	21.69 ± 0.04$^{\blacktriangle}$
pGreen2-12	7.45 ± 0.15	3.12 ± 0.16	16.78 ± 0.75	27.86 ± 0.38	15.47 ± 0.58	2.23 ± 0.11	20.13 ± 0.25

Only one *AthACBP_RNAi*(Nap) line (pGreen1) had significantly altered FA composition of seed oil - an increase in 18:3 and VLC-FA (20:0 and 20:1) at the expense of 18:1 and 18:2. Also, 18:0 levels increased in pGreen1 compared to WT. Overall, the percentage of SFA in seed oil increased from 13.23 in WT to 15.32 in pGreen1, at the expense of PUFA (Table 5.4). The proportion of MUFA in the seed oil of the pGreen1 line remained unchanged compared to WT. Statistical analysis performed on mol% values produced similar results as the analysis with %weight (Appendix 2). Since only one out of 12 lines transformed with pGreen-*AthACBP*_RNAi displayed changes in FA composition of seed oil, further analysis of this line is required to identify what caused this phenotype.

Table 5.4. Composition of FA classes in *A. thaliana* T_2 seeds transformed with *AthACBP-RNAi*(Nap) construct. Mean %weight $\pm$ SD of the means. ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05

Construct	SFA	MUFA	PUFA
WT	13.23 ± 0.06	42.22 ± 0.26	44.53 ± 0.27
pGreen1	15.32 ± 0.09$^{\blacktriangle}$	41.98 ± 0.06	42.69 ± 0.07$^{\blacktriangledown}$
pGreen2-12	12.80 ± 0.39	42.19 ± 0.80	44.99 ± 0.47

5.4 Discussion

Knock-out mutants and RNAi lines have been analyzed for three members of *A. thaliana* ACBP family: ACBP2, ACBP4, and ACBP6 (Mishra *et al.*, 2004; Xiao *et al.*, 2008b; Chen *et al.*, 2008; Kojima *et al.*, 2007). No data has been generated, however, about the effect of ACBP down-regulation on seed oil biosynthesis in these lines. The only phenotype described was of *B. napus* cv. Tantal transformed with the antisense *AthACBP* sequence, showing changes in FA composition of seed oil (Enikeev and Mishutina, 2005).

We produced *A. thaliana* RNAi lines transformed with hairpin constructs for constitutive (pSBS4148 and pSBS4149) and seed specific (pGreen-AthACBP_RNAi) suppression of *AthACBP* (At1g31812) expression. In lines expressing *AthACBP_RNAi*(cons) constructs, the degree of *AthACBP* down-regulation was higher in vegetative tissue (green leaves) than in developing siliques. About half of the analyzed lines displayed *AthACBP* transcript levels lower than 50% of the WT level. *AthACBP* expression in developing seeds decreased to 25-40% of the level in WT seed, when the hairpin construct was expressed under the seed specific napin promoter.

The main change in the FA composition of seed oil of *AthACBP_RNAi*(cons) lines, an increase in 18:1, suggests that there was a build-up of oleic acid in developing seeds that did not get modified on the ER membranes. An increase in 18:1 and 18:2, and a decrease in 18:0 and α-18:3 are similar to the changes observed in *A. thaliana* T_2 and T_3 seed transformed with *BnACBP* constructs having a single chain D9 antibody fusion partner. The

mechanism for such a modification cannot be fully explained. It is possible that the decrease in ACBP levels delays the release of *de novo* FAs from the plastid, which in part can explain the higher degree of desaturation from 18:0 to 18:1. Another process that could have been affected in *AthACBP_RNAi*(cons) seeds is the acyl exchange between acyl-CoA and PC pools, since we have previously shown that changes in ACBP concentrations can affect lysophosphatidylcholine acyltransferase activity. FA elongation from 18:1 to 20:1 seems to be affected in *AthACBP_RNAi*(cons) seeds only to a small extent.

Most of the analyzed *A. thaliana* lines transformed with seed-specific hairpin construct displayed no changes in FA composition of seed oil. A few possible reasons could explain this lack of the phenotype in *AthACBP_RNAi*(Nap) lines. First, levels of *AthACBP* transcript may not closely reflect the AthACBP protein levels in developing seeds. Second, other members of *A. thaliana* ACBP family could compensate for the decrease in At1g31812 gene transcript levels. Identification and analysis of the homozygous *AthACBP* knock-out line may prove to be more effective in studying the role of ACBP on seed oil biosynthesis. Also, a mutant of the multiple *ACBP* genes might provide a useful tool for studying the role(s) of ACBP family in plant metabolism.

6. General Discussion

6.1 Summary

Small cytosolic acyl-CoA binding protein (ACBP) was selected as a subject of investigation in this study with the main focus on examining the physiological role of this protein in seed oil biosynthesis. The ubiquitous expression of ACBP and its ability to bind acyl-CoA with high affinity, combined with the described functions of the maintenance and intracellular transport of acyl-CoA pool, suggest that ACBP may play an important role in metabolic processes in which acyl-CoA is actively involved. Understanding the mechanisms involved in the maintenance, distribution and channelling of acyl-CoAs during seed development could assist efforts toward modification of seed oil content and fatty acid (FA) composition. We applied various strategies including overexpression or down-regulation of *ACBP* in developing seeds of *A. thaliana,* as well as *in vitro* studies of the effects of recombinant ACBP on the enzymatic activities involved in seed oil biosynthesis.

Overexpression of *BnACBP* in *A. thaliana* developing seeds resulted in significant changes in FA composition of acyl-CoA pool and seed oil. The acyl-CoA pool of the transgenic developing seeds had an increase in the proportion of saturated and some unsaturated (C16:0, C18:0 and C18:2) acyl groups at the expense of very-long chain saturated and monounsaturated groups (C20:0, C20:1 and C22:1). Similar changes in the FA composition of seed oil were observed, where the percentage of polyunsaturated FA (PUFA) increased, while levels of

very-long monounsaturated FA (MUFA) decreased. Increased levels of saturated FA in the acyl-CoA pool did not translate into similar changes in FA composition of seed oil. The extent of the observed changes in transgenic seeds depended on the structure of the genetic construct used for transformation. Additional sequences, such as in-frame fusions with oleosin variants and targeting signals, affected the protein levels and subcellular localization of the transgene's product in developing seeds. Overall, the results of analysis of the transgenic seeds expressing *BnACBP* constructs led us to propose that ACBP levels in developing seeds might have important role in partitioning of acyl-CoA between FA elongation and desaturation. Because FA desaturation takes place on a phospholipid substrate, our next step was to investigate the effect of ACBP levels on acyl exchange between acyl-CoA and phosphatidylcholine (PC) pools.

Lysophosphatidylcholine acyltransferase (LPCAT), an enzyme involved in acyl editing, was assayed *in vitro* in the presence of different amounts of the recombinant *B. napus* ACBP (rBnACBP). Incorporation of 18:1 from acyl-CoA into PC and transfer of 18:2 from PC into acyl-CoA pool were enhanced by ACBP in forward and reverse reactions of LPCAT assayed in plant or yeast microsomes. An increased rate of 18:1 esterification to PC creates more substrate for FAD2 desaturase, which could result in higher production of 18:2, which can get desaturated further to 18:3 by FAD3. Results of the LPCAT assays in combination with the data from acyl-CoA pool analysis of *A. thaliana* seeds expressing *BnACBP* suggest that 10 kDa ACBP is involved in acyl editing by enhancing LPCAT activity in forward and reverse reactions. This facilitates the

removal of PUFA from PC, enriching the acyl-CoA pool with PUFA and producing endogenous LPC that can be re-acylated with *de novo* synthesized 18:1. Results of the *in vitro* assays also suggested that ACBP may have some moderate effect on acyltransferase activities of the Kennedy pathway (*sn*-glycerol-3-phosphate acyltransferase and diacylglycerol acyltransferase), in a manner dependent on the ratio of ACBP to acyl-CoA in the assay and the source of the microsome material used.

The suppression of *ACBP* expression in *A. thaliana* was attempted through RNA interference (RNAi) technology. Plants transformed with the constitutively expressed hairpin RNA constructs displayed significant changes in FA composition of seed oil. An increase in the 18:1 percentage in seed oil of RNAi lines suggested that a decrease in the levels of the small cytosolic ACBP might have caused a build-up of oleic acid in the acyl-CoA pool due to reduction of the acyl editing rates.

Collectively, the results of the study suggest that the small cytosolic ACBP plays an important role in acyl exchange between acyl-CoA and PC pools. The turnover of acyl groups (acyl editing) on PC catalyzed by LPCAT enhances channelling of the PUFAs into the acyl-CoA pool and subsequently into the seed oil.

6.2 Limitations of the study

The use of *A. thaliana* in this study offered numerous advantages. First, *A. thaliana* accumulates seed oil, which makes it an adequate model system for studying seed oil formation. It belongs to the Crucifer family, which includes

commercial oilseed crops such as *B. napus*. *Agrobacterium*-mediated transformation of *A. thaliana* is fast and technically simple (floral dip) and usually results in a high number of transgenic lines. The life cycle of the plant is rapid (about 6 weeks from germination to mature seeds), which allows analysis of multiple generations in a relatively short time. Also, extensive genetic and genomic recourses available for *A. thaliana*, including full genome sequence and insertional mutant collections, facilitate the analysis of gene functions. On the other hand, the small seed size and relatively small amount of seeds per plant impose certain technical challenges on seed oil analysis. A small sample size of 10 mg was used for seed oil analysis in this study in order to provide sufficient number of technical replicates, and to preserve some seeds for propagation or other type of analysis. Using such a small sample size affects the accuracy of the measurements, and makes the quantitative analysis, like determining seed oil content, challenging. This problem can partially be addressed by using multiple analytical methods (gravimetry and gas chromatography) in determining seed oil content. Transformation of *B. napus* provides an alternative which overcomes the problem of small sample size. In this study, however, we obtained only one *B. napus* transformant, which was insufficient to draw reliable conclusions of the effect of *ACBP* overexpression on seed oil content in this crop.

In vitro studies of the effect of rBnACBP on the acyltransferase activities were performed using one type of acyl-CoA substrate, [1-^{14}C]18:1-CoA. Using other acyl-CoA species in combination with rACBP in the acyltransferase assays may reveal whether ACBP can affect the selectivity or specificity of the enzymes

of glycerolipid biosynthesis. Liver ACBP has been reported to modulate the selectivity of GPAT in rat liver microsomes (Jolly *et al.*, 2000; Kannan *et al.*, 2003). It would be interesting to find out if ACBP has a role in determining the substrate selectivity of the acyltransferases of seed oil formation.

Transgenic *A. thaliana* plants used for studying the effects of the altered ACBP levels in developing seed on seed oil formation were grown only under one set of growth conditions. Using multiple treatment levels of temperature, light or water supply may reveal different effects or different extents of the effects of ACBP on seed oil formation. The effect of the environmental conditions on the phenotype of *A. thaliana* ACBP knock-outs or over-expressors has been previously demonstrated (Mishra *et al.*, 2004; Chen *et al.*, 2008).

Utilization of the RNAi technology in this study resulted in only partial *ACBP* silencing in *A. thaliana* developing seeds. Identification and analysis of the homozygous *ACBP* knock-out lines may prove to be a more efficient reverse genetics approach to studying ACBP's role in seed oil biosynthesis. Also, down-regulation of the multiple ACBP genes within a plant or a plant tissue might provide a useful tool for studying the role(s) of the ACBP family in plant metabolism.

Additional insight into the effect of overexpressing *ACBP* or suppressing *ACBP* expression on the regulation of lipid synthesis and other aspects of plant metabolism could come from analysis of the transcriptome of developing seed from transgenic plants. For example, Sharma *et al.* (2008) have demonstrated that the overexpression of the gene encoding type 1 DGAT in developing seeds of *B.*

napus affected the expression of numerous other genes including genes encoding enzymes in the *sn*-glycerol-3-phosphate pathway leading to triacylglycerol.

6.3 Future directions in studying ACBP

BSA and ACBP enhance solubilization of acyl-CoAs in aqueous media, and can similarly modulate the activity of some enzymes *in vitro* (LPCAT reverse reaction, DGAT, see Chapter 4). Other activities (LPCAT forward reaction, GPAT) seemed to have rather opposite responses to the addition of BSA or rBnACBP to the reaction mixture. This suggests that, despite the similarity in ligand binding, there might be other factors that differentiate the effect of BSA and rBnACBP on some enzymes. It is tempting to speculate that BnACBP may have some established protein-protein interactions with the enzymes involved in acyl-CoA metabolism. BSA, on the other hand, being a mammalian protein might be lacking the ability to make those interactions with the plant enzymes. Protein-protein interactions have been described for larger Mr members of the *A. thaliana* ACBP family (Li and Chye, 2004; Li *et al.*, 2008; Gao *et al.*, 2008). It may prove useful to explore potential protein-protein interactions between small Mr cytosolic ACBP and other cellular proteins/enzymes using a biophysical or proteomics-based approach.

Another aspect of ACBP functionality that is not yet understood is the mechanism of the intracellular transport of acyl-CoA by small cytosolic ACBP. It appears that the nature of this transport is directed rather than random. Electrostatic forces have been shown to be an important factor in directing ACBP orientation and initial interaction with the membranes (Vallejo *et al.*, 2008). What

factors and long-range stimuli are involved in targeting of the acyl-CoA transport by ACBP still remains unknown.

The sequence and the structure of acyl-CoA binding domain are characterized by a high degree of similarity among ACBPs from different species. Some amino acids within the binding pocket that are directly involved in ligand interactions are highly conserved (Kragelund *et al.*, 1999; Faergeman *et al.*, 2007). Even a single amino acid substitution of these conserved residues severely affected the ligand binding of the protein (Chye *et al.*, 2000; Leung *et al.*, 2004). Other amino acids of the binding domain that vary among different ACBPs have a role determining the shape of the binding site, and thus affect the ligand specificity of the protein (van Aalten *et al.*, 2001; Leung *et al.*, 2006). Understanding the relationship between amino acid sequence and the ligand specificity of ACBP may enable molecular engineering of the acyl-CoA binding sites with desired ligand preferences. Designing ACBP variants that can bind unusual acyl-CoAs may improve the outcome of efforts to produce unusual FAs (hydroxyl-, very long-chain FA) in seed oil of commercial crops.

6.4 Possible applications of the findings

Overexpression of small cytosolic ACBP in developing seeds has been proposed as a molecular tool for modification of FA composition of seed oil (USA patent of Yurchenko *et al.*, 2008). Use of protein fusion partners that can affect subcellular targeting and retention, as well as stability and accumulation of ACBP in developing seeds, can to a certain degree determine the effect of the ACBP transformation on the seed oil formation. In this study, the fusion of ACBP

with oleosin variants significantly enhanced accumulation of recombinant ACBP in developing seeds. Introduction of the signal sequences involved in sub-cellular targeting of the protein may facilitate or re-direct the acyl-CoA transport by ACBP in the cell.

In vitro assays performed in this and previous studies (Brown *et al.*, 1998; Brown *et al.*, 2002) demonstrated that recombinant ACBP could modulate activity of the acyltransferases involved in seed oil biosynthesis depending on the ratio of the binding protein to acyl-CoA in the reaction mixture. It has been proposed that the *in vivo* ACBP: acyl-CoA ratio is close to 1, which means the levels of free acyl-CoA are kept at low nanomolar levels (Rasmussen *et al.*, 1993; Fox *et al.*, 2000a). Thus, using acyl-CoA in complex with its native buffering agent – ACBP – might be more desirable and representative of the *in vivo* situation in contrast to utilization of free acyl-CoA for *in vitro* studies of enzymatic activities that require acyl-CoA substrates.

Overall, this study has contributed to understanding of the fundamental role of ACBP as a mediator in acyl-CoA metabolism, and explored new ways of improvement of the oilseed crops using the small cytosolic ACBP.

7. Reference List

Abell, B.M., High, S. and Moloney, M. M. (2002) Membrane protein topology of oleosin is constrained by its long hydrophobic domain. *J. Biol. Chem.,* **277**(10), 8602-8610.

Abell, B.M., Hahn, M., Holbrook, L. A. and Moloney, M. M. (2004) Membrane topology and sequence requirements for oil body targeting of oleosin. *Plant J.,* **37**(4), 461-470.

Alonso, J.M. and Ecker, J. R. (2006) Moving forward in reverse: Genetic technologies to enable genome-wide phenomic screens in arabidopsis. *Nat. Rev. Genet.,* **7**(7), 524-536.

Banas, A., Dahlqvist, A., Stahl, U., Lenman, M. and Stymne, S. (2000) The involvement of phospholipid:diacylglycerol acyltransferases in triacylglycerol production. *Biochem. Soc. Trans.,* **28**(6), 703-705.

Banhegyi, G., Csala, M., Mandl, J., Burchell, A., Burchell, B., Marcolongo, P., Fulceri, R. and Benedetti, A. (1996) Fatty acyl-CoA esters and the permeability of rat liver microsomal vesicles. *Biochem. J.,* **320 (Pt 1)**(Pt 1), 343-344.

Bates, P.D., Durrett, T. P., Ohlrogge, J. B. and Pollard, M. (2009) Analysis of acyl fluxes through multiple pathways of triacylglycerol synthesis in developing soybean embryos. *Plant Physiol.,* **150**(1), 55-72.

Beaudoin, F. and Napier, J. A. (2000) The targeting and accumulation of ectopically expressed oleosin in non-seed tissues of *Arabidopsis thaliana*. *Planta,* **210**(3), 439-445.

Beaudoin, F., Lacey, D. J. and Napier, J. A. (1999) The biogenesis of the plant seed oil body: Oleosin protein is synthesised by ER-bound ribosomes. *Plant Physiology and Biochemistry,* **37**(6), 481-490.

Bernerth, R. and Frentzen, M. (1990) Utilization of erucoyl-CoA by acyltransferases from developing seeds of *Brassica napus* (L.) involved in triacylglycerol biosynthesis. *Plant Science,* **67**(1), 21-28.

Bhuiyan J., Pritchard, P. H., Pande, S. V. and Seccombe, D. W. (1995) Effects of high-fat diet and fasting on levels of acyl-coenzyme A binding protein in liver, kidney, and heart of rat. *Metab. Clin. Exp.,* **44**, 1185.

Blacklock, B.J. and Jaworski, J. G. (2006) Substrate specificity of *Arabidopsis* 3-ketoacyl-CoA synthases. *Biochemical and Biophysical Research Communications,* **346**(2), 583-590.

Bligh, E.G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.,* **37**(8), 911-917.

Boord, J.B., Fazio, S. and Linton, M. F. (2002) Cytoplasmic fatty acid-binding proteins: Emerging roles in metabolism and atherosclerosis. *Curr. Opin. Lipidol.,* **13**(2), 141-147.

Borboni, P., Condorelli, L., De Stefanis, P., Sesti, G. and Lauro, R. (1991) Modulation of insulin secretion by diazepam binding inhibitor and its processing products. *Neuropharmacology,* **30**(12B), 1399-1403.

Boylan, J.G. and Hamilton, J. A. (1992) Interactions of acyl-coenzyme A with phosphatidylcholine bilayers and serum albumin. *Biochemistry,* **31**(2), 557-567.

Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.,* **72**, 248.

Brown, A.P., Slabas, A. R. and Denton, H. (2002) Substrate selectivity of plant and microbial lysophosphatidic acid acyltransferases. *Phytochemistry,* **61**(5), 493-501.

Brown, A.P., Johnson, P., Rawsthorne, S. and Hills, M. J. (1998) Expression and properties of acyl-CoA binding protein from *Brassica napus*. *Plant Physiology and Biochemistry,* **36**(9), 629-635.

Brown, J.R. and Shockley, P. (1982) Serum albumin: Structure and characterisation of its ligand binding sites. In *Lipid-Protein Interactions* (P. Jost and O.H. Griffith, eds) New York: Wiley & Sons.

Browse, J., McConn, M., James, D.,Jr and Miquel, M. (1993) Mutants of *Arabidopsis* deficient in the synthesis of alpha-linolenate. Biochemical and genetic characterization of the endoplasmic reticulum linoleoyl desaturase. *J. Biol. Chem.,* **268**(22), 16345-16351.

Burton, M., Rose, T. M., Faergeman, N. J. and Knudsen, J. (2005) Evolution of the acyl-CoA binding protein (ACBP). *Biochem. J.,* **392**, 299-307.

Büttner, M. and Singh, K. B. (1997) *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. *Proceedings of the National Academy of Sciences of the United States of America,* **94**(11), 5961-5966.

Cahoon, E.B., Shockey, J. M., Dietrich, C. R., Gidda, S. K., Mullen, R. T. and Dyer, J. M. (2007) Engineering oilseeds for sustainable production of industrial and nutritional feedstocks: Solving bottlenecks in fatty acid flux. *Curr. Opin. Plant Biol.,* **10**(3), 236-244.

Cases, S., Smith, S. J., Zheng, Y., Myers, H. M., Lear, S. R., Sande, E., Novak, S., Collins, C., Welch, C. B., Lusis, A. J., Erickson, S. K. and 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 of the United States of America,* **95**(22), 13018-13023.

Chao H., Martin G., Russell, W. K., Waghela, S. D., Russell, D. H., Schroeder F. and Kier, A. B. (2002) Membrane charge and curvature determine interaction with acyl CoA binding protein (ACBP) and fatty acyl CoA targeting. *Biochemistry (N. Y.),* **41**, 10540.

Chao, H., Zhou, M., McIntosh, A., Schroeder, F. and Kier, A. B. (2003) ACBP and cholesterol differentially alter fatty acyl CoA utilization by microsomal ACAT. *J. Lipid Res.,* **44**(1), 72-83.

Chen Z.-W., Agerberth B., Gell K., Andersson M., Mutt V., Ostenson C.-G., Efendic S., Barros-Soderling J., Persson B., Jornvall H. (1988) Isolation and characterization of porcine diazepam-binding inhibitor, a polypeptide not only of cerebral occurrence but also common in intestinal tissues and with effects on regulation of insulin release. *Eur. J. Biochem.,* **174**, 239.

Chen, Q.F., Xiao, S. and Chye, M. L. (2008) Overexpression of the *Arabidopsis* 10-kilodalton acyl-coenzyme A-binding protein ACBP6 enhances freezing tolerance. *Plant Physiol.,* **148**(1), 304-315.

Chmurzynska, A. (2006) The multigene family of fatty acid-binding proteins (FABPs): Function, structure and polymorphism. *J. Appl. Genet.,* **47**(1), 39-48.

Choi, J., Stukey, J., Hwang, S. and Martin, C. E. (1996) Regulatory elements that control transcription activation and unsaturated fatty acid-mediated repression of the *Saccharomyces cerevisiae* OLE1 gene. *J. Biol. Chem.,* **271**(7), 3581-3589.

Choinowski, T., Hauser, H. and Piontek, K. (2000) Structure of sterol carrier protein 2 at 1.8 A resolution reveals a hydrophobic tunnel suitable for lipid binding. *Biochemistry,* **39**(8), 1897-1902.

Christie, W., Nikolova-Damyanova, B., Laakso, P. and Herslof, B. (1991) Stereospecific analysis of triacyl-sn-glycerols via resolution of diastereomeric diacylglycerol derivatives by high-performance liquid chromatography on silica. *J. Am. Oil Chem. Soc.,* **68**(10), 695-701.

Chye, M.L. (1998) *Arabidopsis* cDNA encoding a membrane-associated protein with an acyl-CoA binding domain. *Plant Mol. Biol.,* **38**(5), 827-838.

Chye, M.L., Li, H. Y. and Yung, M. H. (2000) Single amino acid substitutions at the acyl-CoA-binding domain interrupt 14[C]palmitoyl-CoA binding of ACBP2, an *Arabidopsis* acyl-CoA-binding protein with ankyrin repeats. *Plant Mol. Biol.,* **44**(6), 711-721.

Chye, M.L., Huang, B. Q. and Zee, S. Y. (1999) Isolation of a gene encoding *Arabidopsis* membrane-associated acyl-CoA binding protein and immunolocalization of its gene product. *Plant J.,* **18**(2), 205-214.

Clough, S.J. and Bent, A. F. (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.,* **16**(6), 735-743.

Coe, N.R. and Bernlohr, D. A. (1998) Physiological properties and functions of intracellular fatty acid-binding proteins. *Biochimica Et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* **1391**(3), 287-306.

Cohen Simonsen, A., Bernchou Jensen, U., Fmrgeman, N. J., Knudsen, J. and Mouritsen, O. G. (2003) Acyl-coenzyme A organizes laterally in membranes and is recognized specifically by acyl-coenzyme A binding protein. *FEBS Lett.,* **552**(2-3), 253-258.

Curry, S., Brick, P. and Franks, N. P. (1999) Fatty acid binding to human serum albumin: New insights from crystallographic studies. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* **1441**(2-3), 131-140.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., Scheible, W. R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology*, **139**(1), 5-17.

Dahlqvist, A., Ståhl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H. and 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 of the United States of America,* **97**(12), 6487-6492.

Das, S., Castillo, C. and Stevens, T. (2001) Phospholipid remodeling/generation in *Giardia*: The role of the lands cycle. *Trends in Parasitology,* **17**(7), 316-319.

Davies, K.M. (2007) Genetic modification of plant metabolism for human health benefits. *Mutat. Res.,* **622**(1-2), 122-137.

Diakou, P., Fedou, S., Carde, J., Cassagne, C., Moreau, P. and Maneta-Peyret, L. (2002) Immunolocalization of long-chain acyl-CoAs in plant cells. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* **1583**(1), 85-90.

DiRusso, C., Heimert, T. and Metzger, A. (1992) Characterization of FadR, a global transcriptional regulator of fatty acid metabolism in *Escherichia coli*. interaction with the fadB promoter is prevented by long chain fatty acyl coenzyme A. *J. Biol. Chem.,* **267**(12), 8685-8691.

Domergue, F., Chevalier, S., Creach, A., Cassagne, C. and Lessire, R. (2000) Purification of the acyl-CoA elongase complex from developing rapeseed and characterization of the 3-ketoacyl-CoA synthase and the 3-hydroxyacyl-CoA dehydratase. *Lipids,* **35**(5), 487-494.

Douliez, J.P., Pato, C., Rabesona, H., Molle, D. and Marion, D. (2001) Disulfide bond assignment, lipid transfer activity and secondary structure of a 7-kDa plant lipid transfer protein, LTP2. *Eur. J. Biochem.,* **268**(5), 1400-1403.

Dunphy, J.T., Schroeder, H., Leventis, R., Greentree, W. K., Knudsen, J. K., Silvius, J. R. and Linder, M. E. (2000) Differential effects of acyl-CoA binding protein on enzymatic and non-enzymatic thioacylation of protein and peptide substrates. *Biochim. Biophys. Acta,* **1485**(2-3), 185-198.

Dunphy, J.T. and Linder, M. E. (1998) Signalling functions of protein palmitoylation. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* **1436**(1-2), 245-261.

Ecker, J.R. and Davis, R. W. (1986) Inhibition of gene expression in plant cells by expression of antisense RNA. *Proc. Natl. Acad. Sci. U. S. A.,* **83**(15), 5372-5376.

Edqvist, J., Ronnberg, E., Rosenquist, S., Blomqvist, K., Viitanen, L., Salminen, T. A., Nylund, M., Tuuf, J. and Mattjus, P. (2004) Plants express a lipid transfer protein with high similarity to mammalian sterol carrier protein-2. *J. Biol. Chem.,* **279**(51), 53544-53553.

Elholm, M., Garras, A., Neve, S., Tornehave, D., Lund, T. B., Skorve, J., Flatmark, T., Kristiansen, K. and Berge, R. K. (2000) Long-chain acyl-CoA esters and acyl-CoA binding protein are present in the nucleus of rat liver cells. *J. Lipid Res.,* **41**(4), 538-545.

Engeseth, N.J., Pacovsky, R. S., Newman, T. and Ohlrogge, J. B. (1996) Characterization of an acyl-CoA-binding protein from *Arabidopsis thaliana*. *Arch. Biochem. Biophys.,* **331**(1), 55-62.

Enikeev, A.G. and Mishutina, U. O. (2005) Physiological effects of rapeseed transformation with the *acb* gene as affected by the genetic vector structure. **52**(5), 668-671.

Erber, A., Horstmann, C. and Schobert, C. (1997) A cDNA clone for acyl-CoA-binding protien from castor bean. *Plant Physiology,* **114**, 396-396.

Eto, J., Suzuki, Y., Ohkawa, H. and Yamaguchi, I. (2003) Anti-herbicide single-chain antibody expression confers herbicide tolerance in transgenic plants. *FEBS Lett.,* **550**(1-3), 179-184.

Faergeman, N.J. and Knudsen J. (1997) Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem. J.,* **323**, 1.

Faergeman, N.J., Sigurskjold, B. W., Kragelund, B. B., Andersen, K. V. and Knudsen J. (1996) Thermodynamics of ligand binding to acyl-coenzyme A binding protein studied by titration calorimetry. *Biochemistry (N. Y.),* **35**, 14118.

Faergeman, N.J., Wadum, M., Feddersen, S., Burton, M., Kragelund, B. B. and Knudsen, J. (2007) Acyl-CoA binding proteins; structural and functional conservation over 2000 MYA. *Mol. Cell. Biochem.,* **299**, 55-65.

Faergeman, N.J., Feddersen, S., Christiansen, J. K., Larsen, M. K., Schneiter, R., Ungermann, C., Mutenda, K., Roepstorff, P. and Knudsen, J. (2004) Acyl-CoA-binding protein, Acb1p, is required for normal vacuole function and ceramide synthesis in *Saccharomyces cerevisiae*. *Biochem. J.,* **380**, 907-918.

Falk, K.L. and Guerra, D. J. (1993) Coenzyme A biosynthesis in plants: Partial purification and characterization of pantothenate kinase from spinach. *Arch. Biochem. Biophys.,* **301**(2), 424-430.

Fasano, M., Curry, S., Terreno, E., Galliano, M., Fanali, G., Narciso, P., Notari, S. and Ascenzi, P. (2005) The extraordinary ligand binding properties of human serum albumin. *IUBMB Life,* **57**(12), 787-796.

Fisher, E., Nitz, I., Gieger, C., Grallert, H., Gohlke, H., Lindner, I., Dahm, S., Boeing, H., Burwinkel, B., Rathmann, W., Wichmann, H. E., Schrezenmeir, J., Illig, T. and Doring, F. (2007) Association of acyl-CoA-binding protein (ACBP) single nucleotide polymorphisms and type 2 diabetes in two german study populations. *Mol. Nutr. Food Res.,* **51**(2), 178-184.

Fox, S.R., Rawsthorne, S. and Hills, M. J. (2000a) Role of acyl-CoAs and acyl-CoA-binding protein in regulation of carbon supply for fatty acid biosynthesis. *Biochem. Soc. Trans.,* **28**(6), 672-674.

Fox, S.R., Hill, L. M., Rawsthorne, S. and Hills, M. J. (2000b) Inhibition of the glucose-6-phosphate transporter in oilseed rape (*Brassica napus* L.) plastids by acyl-CoA thioesters reduces fatty acid synthesis. *Biochem. J.,* **352 Pt 2**, 525-532.

Franch J., Knudsen J., Ellis, B. A., Pedersen, P. K., Cooney, G. J. and Jensen J. (2002) Acyl CoA binding protein expression is fiber type-specific and elevated in muscles from the obese insulin-resistant Zucker rat. *Diabetes,* **51**, 449.

Frolov A., Cho, T. H., Billheimer, J. T. and Schroeder F. (1996) Sterol carrier protein-2, a new fatty acyl coenzyme A-binding protein. *J. Biol. Chem.,* **271**, 31878.

Frolov, A.A. and Schroeder F. (1998) Acyl coenzyme A binding protein: Conformational sensitivity to long chain fatty acyl-CoA. *J. Biol. Chem.,* **273**, 11049.

Furukawa-Stoffer, T.L., Boyle, R. M., Thomson, A. L., Sarna, M. A. and Weselake, R. J. (2003) Properties of lysophosphatidylcholine acyltransferase from *Brassica napus* cultures. *Lipids,* **38**(6), 651-656.

Fyrst, H., Knudsen, J., Schott, M. A., Lubin, B. H. and Kuypers, F. A. (1995) Detection of acyl-CoA-binding protein in human red blood cells and investigation of its role in membrane phospholipid renewal. *Biochem. J.,* **306 (Pt 3)**, 793-799.

Gaigg B., Neergard, T. B., Schneiter R., Hansen, J. K., Faergeman, N. J., Jensen, N. A., Andersen, J. R., Friis J., Sandhoff K. and Knudsen J. (2001) Depletion of acyl CoA binding protein affects sphingolipid synthesis and causes vesicle accumulation and membrane defects in *S. cerevisiae*. *Mol. Biol. Cell,* **12**, 1147.

Gao, W., Xiao, S., Li, H. Y., Tsao, S. W. and Chye, M. L. (2008) *Arabidopsis thaliana* acyl-CoA-binding protein ACBP2 interacts with heavy-metal-binding farnesylated protein AtFP6. *New Phytol.,* **181**(1), 89-102.

García-Olmedo, F., Molina, A., Segura, A. and Moreno, M. (1995) The defensive role of nonspecific lipid-transfer proteins in plants. *Trends Microbiol.,* **3**(2), 72-74.

Ghosal, A., Banas, A., Ståhl, U., Dahlqvist, A., Lindqvist, Y. and Stymne, S. (2007) *Saccharomyces cerevisiae* phospholipid:diacylglycerol acyl transferase (PDAT) devoid of its membrane anchor region is a soluble and active enzyme retaining its substrate specificities. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* **1771**(12), 1457-1463.

Glatz, J.F. and Veerkamp, J. H. (1983) A radiochemical procedure for the assay of fatty acid binding by proteins. *Anal. Biochem.,* **132**(1), 89-95.

Glick, B.S. and Rothman, J. E. (1987) Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature,* **326**, 309.

Gossett, R.E., Frolov, A. A., Roths, J. B., Behnke, W. D., Kier, A. B. and Schroeder F. (1996) Acyl co A binding proteins: Multiplicity and function. *Lipids,* **31**, 895.

Graham, I.A., Larson, T. and Napier, J. A. (2007) Rational metabolic engineering of transgenic plants for biosynthesis of omega-3 polyunsaturates. *Curr. Opin. Biotechnol.,* **18**(2), 142-147.

Graham, I.A. (2008) Seed storage oil mobilization. *Annual Review of Plant Biology,* **59**(1), 115-142.

Guerbette, F., Grosbois, M., Jolliot-Croquin, A., Kader, J. and Zachowski, A. (1999) Lipid-transfer proteins from plants: Structure and binding properties. *Mol. Cell. Biochem.,* **192**(1), 157-161.

Guerrero, C., Martin-Rufian, M., Reina, J. J. and Heredia, A. (2006) Isolation and characterization of a cDNA encoding a membrane bound acyl-CoA binding protein from *Agave americana* L. epidermis. *Plant Physiol. Biochem.,* **44**(1), 85-90.

Guidotti, A., Forchetti, C. M., Corda, M. G., Konkel, D., Bennett, C. D. and Costa, E. (1983) Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *PNAS,* **80**(11), 3531-3535.

Haapalainen, A.M., van Aalten, D. M. F., Meriläinen, G., Jalonen, J. E., Pirilä, P., Wierenga, R. K., Hiltunen, J. K. and Glumoff, T. (2001) Crystal structure of the liganded SCP-2-like domain of human peroxisomal multifunctional enzyme type 2 at 1.75 Å resolution. *J. Mol. Biol.,* **313**(5), 1127-1138.

Hansen, J.S., Faergeman, N. J., Kragelund, B. B. and Knudsen, J. (2008) Acyl-CoA-binding protein (ACBP) localizes to the endoplasmic reticulum and golgi in a ligand-dependent manner in mammalian cells. *Biochem. J.,* **410**(3), 463-472.

Hara, A. and Radin, N. S. (1978) Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.,* **90**(1), 420-426.

Hellens, R.P., Edwards, E. A., Leyland, N. R., Bean, S. and Mullineaux, P. M. (2000) pGreen: A versatile and flexible binary ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.,* **42**(6), 819-832.

Helliwell, C. and Waterhouse, P. (2003) Constructs and methods for high-throughput gene silencing in plants. *Methods,* **30**(4), 289-295.

Hertz R., Magenheim J., Berman I. and Bar-Tana J. (1998) Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4a. *Nature,* **392**, 512.

Herzog, M. (1995) In: RNA from seeds. http://www.net/hypermail/ARABIDOPSIS/9504/0017.html. **1995**.

Hills, M.J., Watson, M. D. and Murphy, D. J. (1993) Targeting of oleosins to the oil bodies of oilseed rape (*Brassica napus* L.). *Planta,* **189**(1), 24-29.

Hills, M.J., Dann, R., Lydiate, D. and Sharpe, A. (1994) Molecular cloning of a cDNA from *Brassica napus* L. for a homologue of acyl-CoA-binding protein. *Plant Mol. Biol.,* **25**(5), 917-920.

Hobbs, D.H., Lu, C. and Hills, M. J. (1999) Cloning of a cDNA encoding diacylglycerol acyltransferase from *Arabidopsis thaliana* and its functional expression. *FEBS Lett.,* **452**(3), 145-149.

Huang, A.H. (1996) Oleosins and oil bodies in seeds and other organs. *Plant Physiol.,* **110**(4), 1055-1061.

Huang, A.H.C. (1994) Structure of plant seed oil bodies. *Curr. Opin. Struct. Biol.,* **4**, 493-498.

Huang, H., Atshaves, B. P., Frolov, A., Kier, A. B. and Schroeder, F. (2005) Acyl-coenzyme A binding protein expression alters liver fatty acyl-coenzyme A metabolism. *Biochemistry (N. Y.),* **44**(30), 10282-10297.

Huang, Y.S., Pereira, S. L. and Leonard, A. E. (2004) Enzymes for transgenic biosynthesis of long-chain polyunsaturated fatty acids. *Biochimie,* **86**(11), 793-798.

Ichihara, K., Mae, K., Sano, Y. and Tanaka, K. (1995) 1-acylglycerophosphocholine O-acyltransferase in maturing safflower seeds. *Planta,* **196**(3), 551-557.

Jackson, F.M., Fraser, T. C., Smith, M. A., Lazarus, C., Stobart, A. K. and Griffiths, G. (1998) Biosynthesis of C18 polyunsaturated fatty acids in microsomal membrane preparations from the filamentous fungus *Mucor circinelloides*. *Eur. J. Biochem.,* **252**(3), 513-519.

Jaworski, J.G. (1987) Biosynthesis of monoenoic and polyenoic fatty acids. In *The Biochemistry of Plants* Vol.9 edn. (P.K. Stumpt, ed) Academic Press, Inc.

Johnson, P.E., Rawsthorne, S. and Hills, M. J. (2002) Export of acyl chains from plastids isolated from embryos of *Brassica napus* (L.). *Planta,* **215**(3), 515-517.

Johnson, P.E., Fox, S. R., Hills, M. J. and Rawsthorne, S. (2000) Inhibition by long-chain acyl-CoAs of glucose 6-phosphate metabolism in plastids isolated from developing embryos of oilseed rape (*Brassica napus* L.). *Biochem. J.,* **348 Pt 1**, 145-150.

Jolly, C.A., Wilton, D. A. and Schroeder F. (2000) Microsomal fatty acyl CoA transacylation and hydrolysis: Fatty acyl CoA species dependent modulation by liver fatty acyl CoA binding proteins. *Biochim. Biophys. Acta,* **1483**, 185.

Kader, J. (1996) Lipid-transfer proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* **47**(1), 627-654.

Kannan, L., Knudsen, J. and Jolly, C. A. (2003) Aging and acyl-CoA binding protein alter mitochondrial glycerol-3-phosphate acyltransferase activity. *Biochim. Biophys. Acta,* **1631**(1), 12-16.

Kerkhoff, C., Beuck, M., Threige-Rasmussen, J., Spener, F., Knudsen, J. and Schmitz, G. (1997) Acyl-CoA binding protein (ACBP) regulates acyl-CoA:cholesterol acyltransferase (ACAT) in human mononuclear phagocytes. *Biochim. Biophys. Acta,* **1346**(2), 163-172.

Kikuchi, S., Satoh, K., Nagata, T., Kawagashira, N., Doi, K., Kishimoto, N., Yazaki, J., Ishikawa, M., Yamada, H., Ooka, H., Hotta, I., Kojima, K., Namiki, T., Ohneda, E., Yahagi, W., Suzuki, K., Li, C. J., Ohtsuki, K., Shishiki, T., Otomo, Y., Murakami, K., Iida, Y., Sugano, S., Fujimura, T., Suzuki, Y., Tsunoda, Y., Kurosaki, T., Kodama, T., Masuda, H., Kobayashi, M., Xie, Q., Lu, M., Narikawa, R., Sugiyama, A., Mizuno, K., Yokomizo, S., Niikura, J., Ikeda, R., Ishibiki, J., Kawamata, M., Yoshimura, A., Miura, J., Kusumegi, T., Oka, M., Ryu, R., Ueda, M., Matsubara, K., Kawai, J., Carninci, P., Adachi, J., Aizawa, K., Arakawa, T., Fukuda, S., Hara, A., Hashizume, W., Hayatsu, N., Imotani, K., Ishii, Y., Itoh, M., Kagawa, I., Kondo, S., Konno, H., Miyazaki, A., Osato, N., Ota, Y., Saito, R., Sasaki, D., Sato, K., Shibata, K., Shinagawa, A., Shiraki, T., Yoshino, M., Hayashizaki, Y. and Yasunishi, A. (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from *Japonica* rice. *Science,* **301**(5631), 376-379.

Knudsen J., Jensen, M. V., Hansen, J. K., Faergeman, N. J., Neergard T. and Gaigg B. (1999) Role of acyl CoA binding protein in acyl CoA transport, metabolism, and cell signaling. *Mol. Cell. Biochem.,* **192**, 95.

Knudsen J., Faergeman, N. J., Skott H., Hummel R., Borsting C., Rose, T. M., Andersen, J. S., Hojrup P., Roepstorff P. and Kristiansen K. (1994) Yeast acyl-CoA-binding protein: Acyl-CoA-binding affinity and effect on intracellular acyl-CoA pool size. *Biochem. J.,* **302**, 479.

Knudsen, J., Hojrup, P., Hansen, H. O., Hansen, H. F. and Roepstorff, P. (1989) Acyl-CoA-binding protein in the rat: purification, binding characteristics, tissue concentrations and amino acid sequence. *Biochem. J.,* **262**(2), 513-519.

Kojima, M., Casteel, J., Miernyk, J. A. and Thelen, J. J. (2007) The effects of down-regulating expression of *Arabidopsis thaliana* membrane-associated acyl-CoA binding protein 2 on acyl-lipid composition. *Plant Science,* **172**(1), 36-44.

Kolmer, M., Roos, C., Tirronen, M., Myohanen, S. and Alho, H. (1994) Tissue-specific expression of the diazepam-binding inhibitor in *Drosophila melanogaster*: Cloning, structure, and localization of the gene. *Mol. Cell. Biol.,* **14**(10), 6983-6995.

Kragelund, B.B., Knudsen, J. and Poulsen, F. M. (1999) Acyl-coenzyme A binding protein (ACBP). *Biochim. Biophys. Acta,* **1441**(2-3), 150-161.

Kragelund, B.B., Andersen, K. V., Madsen, J. C., Knudsen, J. and Poulsen, F. M. (1993) Three-dimensional structure of the complex between acyl-coenzyme A binding protein and palmitoyl-coenzyme A. *J. Mol. Biol.,* **230**(4), 1260-1277.

Kragelund, B.B., Poulsen, K., Andersen, K. V., Baldursson, T., Kroll, J. B., Neergard, T. B., Jepsen, J., Roepstorff, P., Kristiansen, K., Poulsen, F. M. and Knudsen, J. (1999) Conserved residues and their role in the structure, function, and stability of acyl-coenzyme A binding protein. *Biochemistry (N. Y.),* **38**(8), 2386-2394.

Krysan, P.J., Young, J. C. and Sussman, M. R. (1999) T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell,* **11**(12), 2283-2290.

Kubis, S.E., Pike, M. J., Everett, C. J., Hill, L. M. and Rawsthorne, S. (2004) The import of phosphoenolpyruvate by plastids from developing embryos of oilseed rape, *Brassica napus* (L.), and its potential as a substrate for fatty acid synthesis. *J. Exp. Bot.,* **55**(402), 1455-1462.

Kunst, L. and Samuels, A. L. (2003) Biosynthesis and secretion of plant cuticular wax. *Prog. Lipid Res.,* **42**(1), 51-80.

Kupke, T., Hernandez-Acosta, P. and Culianez-Macia, F. A. (2003) 4'-phosphopantetheine and coenzyme A biosynthesis in plants. *J. Biol. Chem.,* **278**(40), 38229-38237.

Lands, W.E.M. (1960) Metabolism of glycerolipids. II. THE ENZYMATIC ACYLATION OF LYSOLECITHIN. *J. Biol. Chem.,* **235**(8), 2233-2237.

Larsen, M.K., Tuck, S., Faergeman, N. J. and Knudsen, J. (2006) MAA-1, a novel acyl-CoA-binding protein involved in endosomal vesicle transport in *Caenorhabditis elegans*. *Mol. Biol. Cell,* **17**(10), 4318-4329.

Larson, T.R. and Graham, I. A. (2001) A novel technique for the sensitive quantification of fatty acyl CoA esters from plant tissues. *Plant J.,* **25**, 115.

Larsson, O., Deeney, J. T., Bränström, R., Berggren, P. and Corkey, B. E. (1996) Activation of the ATP-sensitive K channel by long chain acyl-CoA. *J. Biol. Chem.,* **271**(18), 10623-10626.

Laurent, P. and Huang, A. H. C. (1992) Organ- and development-specific acyl coenzyme A lysophosphatidate acyltransferases in palm and meadowfoam. *Plant Physiol.,* **99**(4), 1711-1715.

Lee, L., DeBono, C. A., Campagna, D. R., Young, D. C., Moody, D. B. and Fleming, M. D. (2007) Loss of the acyl-CoA binding protein (acbp) results in fatty acid metabolism abnormalities in mouse hair and skin. *J. Invest. Dermatol.,* **127**(1), 16-23.

Leonard, A.E., Pereira, S. L., Sprecher, H. and Huang, Y. (2004) Elongation of long-chain fatty acids. *Prog. Lipid Res.,* **43**(1), 36-54.

Leung, K.C., Li, H. Y., Mishra, G. and Chye, M. L. (2004) ACBP4 and ACBP5, novel *Arabidopsis* acyl-CoA-binding proteins with kelch motifs that bind oleoyl-CoA. *Plant Mol. Biol.,* **55**(2), 297-309.

Leung, K.C., Li, H. Y., Xiao, S., Tse, M. H. and Chye, M. L. (2006) *Arabidopsis* ACBP3 is an extracellularly targeted acyl-CoA-binding protein. *Planta,* **223**(5), 871-881.

Li, H.Y. and Chye, M. L. (2004) *Arabidopsis* acyl-CoA-binding protein ACBP2 interacts with an ethylene-responsive element-binding protein, AtEBP, via its ankyrin repeats. *Plant Mol. Biol.,* **54**(2), 233-243.

Li, H.Y. and Chye, M. L. (2003) Membrane localization of *Arabidopsis* acyl-CoA binding protein ACBP2. *Plant Mol. Biol.,* **51**(4), 483-492.

Li, H.Y., Xiao, S. and Chye, M. L. (2008) Ethylene- and pathogen-inducible *Arabidopsis* acyl-CoA-binding protein 4 interacts with an ethylene-responsive element binding protein. *J. Exp. Bot.,* **59**(14), 3997-4006.

Lísa, M. and Holčapek, M. (2008) Triacylglycerols profiling in plant oils important in food industry, dietetics and cosmetics using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography A,* **1198-1199**, 115-130.

Little, D., Weselake, R., Pomeroy, K., Furukawa-Stoffer, T. and Bagu, J. (1994) Solubilization and characterization of diacylglycerol acyltransferase from microspore-derived cultures of oilseed rape. *Biochem. J.,* **304 (Pt 3)**(Pt 3), 951-958.

Lung, S.C. and Weselake, R. J. (2006) Diacylglycerol acyltransferase: A key mediator of plant triacylglycerol synthesis. *Lipids,* **41**(12), 1073-1088.

Mandrup S., Jepsen R., Skott H., Rosendal J., Hojrup P., Kristiansen K. and Knudsen J. (1993) Effect of heterologous expression of acyl-CoA-binding protein on acyl-CoA level and composition in yeast. *Biochem. J.,* **290**, 369.

Mandrup, S., Andreasen, P. H., Knudsen, J. and Kristiansen, K. (1993a) Genome organization and expression of the rat ACBP gene family. *Mol. Cell. Biochem.,* **123**(1-2), 55-61.

Mandrup, S., Jepsen, R., Skott, H., Rosendal, J., Hojrup, P., Kristiansen, K. and Knudsen, J. (1993b) Effect of heterologous expression of acyl-CoA-binding protein on acyl-CoA level and composition in yeast. *Biochem. J.,* **290 (Pt 2)**, 369-374.

Mandrup, S., Sorensen, R. V., Helledie, T., Nohr, J., Baldursson, T., Gram, C., Knudsen, J. and Kristiansen, K. (1998) Inhibition of 3T3-L1 adipocyte differentiation by expression of acyl-CoA-binding protein antisense RNA. *J. Biol. Chem.,* **273**(37), 23897-23903.

Matsumoto, S., Yoshiga, T., Yokoyama, N., Iwanaga, M., Koshiba, S., Kigawa, T., Hirota, H., Yokoyama, S., Okano, K., Mita, K., Shimada, T. and Tatsuki, S. (2001) Characterization of acyl-CoA-binding protein (ACBP) in the pheromone gland of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.,* **31**(6-7), 603-609.

McCabe, J.B. and Berthiaume, L. G. (1999) Functional roles for fatty acylated amino-terminal domains in subcellular localization. *Mol. Biol. Cell,* **10**(11), 3771-3786.

Metzner, M., Ruecknagel, K. P., Knudsen, J., Kuellertz, G., Mueller-Uri, F. and Diettrich, B. (2000) Isolation and characterization of two acyl-CoA-binding proteins from proembryogenic masses of *Digitalis lanata* Ehrh. *Planta,* **210**(4), 683-685.

Meyer, A., Kirsch, H., Domergue, F., Abbadi, A., Sperling, P., Bauer, J., Cirpus, P., Zank, T. K., Moreau, H., Roscoe, T. J., Zahringer, U. and Heinz, E. (2004) Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *J. Lipid Res.,* **45**(10), 1899-1909.

Miller, K.R. and Cistola, D. P. (1993) Titration calorimetry as a binding assay for lipid-binding proteins. *Mol. Cell. Biochem.,* **123**(1-2), 29-37.

Milne, K.G. and Ferguson, M. A. (2000) Cloning, expression, and characterization of the acyl-CoA-binding protein in African trypanosomes. *J. Biol. Chem.,* **275**(17), 12503-12508.

Milne, K.G., Guther, M. L. and Ferguson, M. A. (2001) Acyl-CoA binding protein is essential in bloodstream form *Trypanosoma brucei*. *Mol. Biochem. Parasitol.,* **112**(2), 301-304.

Miquel, M. and Browse, J. (1992) *Arabidopsis* mutants deficient in polyunsaturated fatty acid synthesis. Biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase. *J. Biol. Chem.,* **267**(3), 1502-1509.

Mishra, G., Ramalingam, S., Lie Ken Jie, M. S. F. and Chye, M. L. (2004) Charasterization of a mutant in *Arabidopsis ACBP2*. 16th International Plant Lipid Symposium, Budapest, Hungary.

Mogensen, I.B., Schulenberg, H., Hansen, H. O., Spener, F. and Knudsen, J. (1987) A novel acyl-CoA-binding protein from bovine liver. Effect on fatty acid synthesis. *Biochem. J.,* **241**(1), 189-192.

Moloney, M.M., Walker, J. M. and Sharma, K. K. (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep.,* **8**(4), 238-242.

Moncecchi, D., Pastuszyn, A. and Scallen, T. (1991) cDNA sequence and bacterial expression of mouse liver sterol carrier protein-2. *J. Biol. Chem.,* **266**(15), 9885-9892.

Murphy, D.J., Cummins, I. and Kang, A. S. (1989) Synthesis of the major oil-body membrane protein in developing rapeseed (*Brassica napus*) embryos. Integration with storage-lipid and storage-protein synthesis and implications for the mechanism of oil-body formation. *Biochem. J.,* **258**(1), 285-293.

Napier, J.A. (2007) The production of unusual fatty acids in transgenic plants. *Annu. Rev. Plant. Biol.,* **58**, 295-319.

Neess, D., Kiilerich, P., Sandberg, M., Helledie, T., Nielsen, R. and Mandrup, S. (2006) ACBP - a PPAR and SREBP modulated housekeeping gene. **284**, 149-157.

Nitz, I., Doring, F., Schrezenmeir, J. and Burwinkel, B. (2005) Identification of new acyl-CoA binding protein transcripts in human and mouse. *Int. J. Biochem. Cell Biol.,* **37**(11), 2395-2405.

Ogiwara, H., Tanabe, T., Nikawa, J. and Numa, S. (1978) Inhibition of rat-liver acetyl-coenzyme-A carboxylase by palmitoyl-coenzyme A. Formation of equimolar enzyme-inhibitor complex. *Eur. J. Biochem.,* **89**(1), 33-41.

Ohlrogge, J. and Browse, J. (1995) Lipid biosynthesis. *Plant Cell,* **7**(7), 957-970.

Ohshima, T., Hayashi, H. and Chino, M. (1990) Collection and chemical composition of pure phloem sap from *Zea mays* L. *Plant Cell Physiol.,* **31**(5), 735-737.

Oikari, S., Ahtialansaari, T., Heinonen, M., Mauriala, T., Auriola, S., Kiehne, K., Folsch, U., Janne, J., Alhonen, L. and Herzig, K. (2008) Downregulation of PPARs and SREBP by acyl-CoA-binding protein overexpression in transgenic rats. *Pflugers Archiv European Journal of Physiology,* **456**(2), 369-377.

Okamoto, T., Shimada, T., Hara-Nishimura, I., Nishimura, M. and Minamikawa, T. (2003) C-terminal KDEL sequence of A KDEL-tailed cysteine proteinase (sulfhydryl-endopeptidase) is involved in formation of KDEL vesicle and in efficient vacuolar transport of sulfhydryl-endopeptidase. *Plant Physiol.,* **132**(4), 1892-1900.

Orr, W., Keller, W. A. and Singh, J. (1986) Induction of freezing tolerance in an embryogenic cell suspension culture of *Brassica napus* by abscisic acid at room temperature. *Journal of Plant Physiology,* **126**, 23-32.

Pacovsky, R.S. (1996) *Arabidopsis thaliana* acyl-CoA-binding protein: Structure, functions, genetics. PhD thesis. Michigan State University.

Paulussen, R.J. and Veerkamp, J. H. (1990) Intracellular fatty-acid-binding proteins. characteristics and function. *Subcell. Biochem.,* **16**, 175-226.

Petrescu, A.D., Payne, H. R., Boedecker, A., Chao, H., Hertz, R., Bar-Tana, J., Schroeder, F. and Kier, A. B. (2003) Physical and functional interaction of acyl-CoA-binding protein with hepatocyte nuclear factor-4 alpha. *J. Biol. Chem.,* **278**(51), 51813-51824.

Pfanner N., Glick, B. S., Arden, S. R. and Rothman, J. E. (1990) Fatty acylation promotes fusion of transport vesicles with golgi cisternae. *J. Cell Biol.,* **110**, 955.

Ramli, U.S., Baker, D. S., Quant, P. A. and Harwood, J. L. (2002) Use of control analysis to study the regulation of plant lipid biosynthesis. *Biochem. Soc. Trans.,* **30**(Pt 6), 1043-1046.

Rapedius, M., Soom, M., Shumilina, E., Schulze, D., Schonherr, R., Kirsch, C., Lang, F., Tucker, S. J. and Baukrowitz, T. (2005) Long chain CoA esters as competitive antagonists of phosphatidylinositol 4,5-bisphosphate activation in kir channels. *J. Biol. Chem.,* **280**(35), 30760-30767.

Rasmussen, J.T., Rosendal, J. and Knudsen, J. (1993) Interaction of acyl-CoA binding protein (ACBP) on processes for which acyl-CoA is a substrate, product or inhibitor. *Biochem. J.,* **292 (Pt 3)**, 907-913.

Rasmussen, J.T., Borchers, T. and Knudsen, J. (1990) Comparison of the binding affinities of acyl-CoA-binding protein and fatty-acid-binding protein for long-chain acyl-CoA esters. *Biochem. J.,* **265**(3), 849-855.

Rasmussen, J.T., Faergeman, N. J., Kristiansen K. and Knudsen J. (1994) Acyl-CoA-binding protein (ACBP) can mediate intermembrane acyl-CoA transport and donate acyl-CoA for beta-oxidation and glycerolipid synthesis. *Biochem. J.,* **299**, 165.

Reddy, A.S., Ranganathan, B., Haisler, R. M. and Swize, M. A. (1996) A cDNA encoding acyl-CoA-binding protein from cotton. *Plant Physiol.,* **111**, 348.

Richards, E.W., Hamm, M. W., Fletcher, J. E. and Otto, D. A. (1990) The binding of palmitoyl-CoA to bovine serum albumin. *Biochimica Et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* **1044**(3), 361-367.

Rose, T., Schultz, E. and Todaro, G. (1992) Molecular cloning of the gene for the yeast homolog (ACB) of diazepam binding Inhibitor/Endozepine/Acyl-CoA-binding protein. *PNAS,* **89**(23), 11287-11291.

Rosendal, J., Ertbjerg, P. and Knudsen, J. (1993) Characterization of ligand binding to acyl-CoA-binding protein. *Biochem. J.,* **290 (Pt 2)**, 321-326.

Sakuth, T., Schobert, C., Pecsvaradi, A., Eichholz, A., Komor, E. and Orlich, G. (1993) Specific proteins in the sieve-tube exudate of *Ricinus commuais* L. seedlings: Separation, characterization and in-vivo labelling. *Planta,* **191**(2), 207-213.

Salcedo, G., Sánchez-Monge, R., Barber, D. and Díaz-Perales, A. (2007) Plant non-specific lipid transfer proteins: An interface between plant defence and human allergy. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* **1771**(6), 781-791.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular cloning: A laboratory manual, 2nd edn.*Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sayanova, O.V., Beaudoin, F., Michaelson, L. V., Shewry, P. R. and Napier, J. A. (2003) Identification of *Primula* fatty acid delta 6-desaturases with n-3 substrate preferences. *FEBS Lett.,* **542**(1-3), 100-104.

Schaffner, A.R. and Sheen, J. (1991) Maize rbcS promoter activity depends on sequence elements not found in dicot rbcS promoters. *Plant Cell,* **3**(9), 997-1012.

Schroeder, F., Petrescu, A. D., Huang, H., Atshaves, B. P., McIntosh, A. L., Martin, G. G., Hostetler, H. A., Vespa, A., Landrock, D., Landrock, K. K., Payne, H. R. and Kier, A. B. (2008) Role of fatty acid binding proteins and long chain fatty acids in modulating nuclear receptors and gene transcription. *Lipids,* **43**(1), 1-17.

Schroeder, F., Atshaves, B. P., McIntosh, A. L., Gallegos, A. M., Storey, S. M., Parr, R. D., Jefferson, J. R., Ball, J. M. and Kier, A. B. (2007) Sterol carrier protein-2: New roles in regulating lipid rafts and signaling. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* **1771**(6), 700-718.

Schwender, J., Shachar-Hill, Y. and Ohlrogge, J. B. (2006) Mitochondrial metabolism in developing embryos of *Brassica napus*. *J. Biol. Chem.,* **281**(45), 34040-34047.

Sharma, N., Anderson, M., Kumar, A., Zhang, Y., Giblin, E. M., Abrams, S., Zaharia, L. I., Taylor, D. and Fobert, P. (2008) Transgenic increases in seed oil content are associated with the differential expression of novel *Brassica*-specific transcripts. *BMC Genomics,* **9**(1), 619.

Shimakata, T. and Stumpf, P. K. (1982) Isolation and function of spinach leaf beta-ketoacyl-[acyl-carrier-protein] synthases. *Proc. Natl. Acad. Sci. U. S. A.,* **79**(19), 5808-5812.

Sjolund, R.D. (1997) The phloem sieve element: A river runs through it. *Plant Cell,* **9**(7), 1137-1146.

Slabas, A.R., Simon, J. W. and Brown, A. P. (2001) Biosynthesis and regulation of fatty acids and triglycerides in oil seed rape. Current status and future trends. *Europear Journal of Lipid Science and Technology,* **103**(7), 455-466.

Snyder, C.L., Yurchenko, O. P., Siloto, R. M. P., Chen, X., Liu, Q., Mietkiewska, E. and Weselake, R. J. (2009) Acyltransferase action in the modification of seed oil biosynthesis. *New Biotechnology,* **In Press, Corrected Proof**.

Solstad, T., Fismen, L., Garberg, H. and Fladmark, K. E. (2008) Identification of a novel phosphorylation site of acyl-CoA binding protein (ACBP) in nodularin-induced apoptotic hepatocytes. *Exp. Cell Res.,* **314**(10), 2141-2149.

Ståhl, U., Stålberg, K., Stymne, S. and Ronne, H. (2008) A family of eukaryotic lysophospholipid acyltransferases with broad specificity. *FEBS Letters,* **582**(2), 305-309.

Stobart K., Mancha M., Lenman M., Dahlqvist A. and Stymne S. (1997) Triacylglycerols are synthesised and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L.) seeds. **203**(1), 58-66.

Stobart, A.K. and Stymne, S. (1985) The interconversion of diacylglycerol and phosphatidylcholine during triacylglycerol production in microsomal preparations of developing cotyledons of safflower (*Carthamus tinctorius* L.). *Biochem. J.,* **232**(1), 217-221.

Storch, J. and McDermott, L. (2009) Structural and functional analysis of fatty acid-binding proteins. *J. Lipid Res.,* **50**(Supplement), S126-131.

Stymne, S. and Stobart, A. K. (1984) Evidence for the reversibility of the acyl-CoA:lysophosphatidylcholine acyltransferase in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons and rat liver. *Biochem. J.,* **223**(2), 305-314.

Stymne, S. and Glad, G. (1981) Acyl exchange between oleoyl-CoA and phosphatidylcholine in microsomes of developing soya bean cotyledons and its role in fatty acid desaturation. *Lipids,* **16**(5), 298-305.

Suzui, N., Nakamura, S. I., Fujiwara, T., Hayashi, H. and Yoneyama, T. (2006) A putative acyl-CoA-binding protein is a major phloem sap protein in rice (*Oryza sativa* L.). *J. Exp. Bot.,***57**(11), 2571-2576.

Taskinen, J.P., van Aalten, D. M., Knudsen, J. and Wierenga, R. K. (2007) High resolution crystal structures of unliganded and liganded human liver ACBP reveal a new mode of binding for the acyl-CoA ligand. *Proteins,* **66**(1), 229-238.

Tassin-Moindrot, S., Caille, A., Douliez, J. P., Marion, D. and Vovelle, F. (2000) The wide binding properties of a wheat nonspecific lipid transfer protein. solution structure of a complex with prostaglandin B2. *Eur. J. Biochem.,* **267**(4), 1117-1124.

Taylor, D.C., Barton, D. L., Rioux, K. P., MacKenzie, S. L., Reed, D. W., Underhill, E. W., Pomeroy, M. K. and Weber, N. (1992) Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore-derived and zygotic embryos of *Brassica napus* L. cv reston. *Plant Physiol.,* **99**(4), 1609-1618.

Tucker, S.J. and Baukrowitz, T. (2008) How highly charged anionic lipids bind and regulate ion channels. *J. Gen. Physiol.,* **131**(5), 431-438.

Vahouny, G.V., Chanderbhan, R., Kharroubi, A., Noland, B. J., Pastuszyn, A. and Scallen, T. J. (1987) Sterol carrier and lipid transfer proteins. *Adv. Lipid Res.,* **22**, 83-113.

Vallejo, D.F., Zamarreño, F., Guérin, D. M. A., Grigera, J. R. and Costabel, M. D. (2009) Prediction of the most favorable configuration in the ACBP–membrane interaction based on electrostatic calculations. *Biochimica Et Biophysica Acta (BBA) - Biomembranes,* **1788**(3), 696-700.

Vallejo, D.F.G., Grigera, J. R. and Costabel, M. D. (2008) A hydrophobic loop in acyl-CoA binding protein is functionally important for binding to palmitoyl-coenzyme A: A molecular dynamics study. *Int. J. Biol. Macromol.,* **42**(3), 271-277.

van Aalten, D.M., Milne, K. G., Zou, J. Y., Kleywegt, G. J., Bergfors, T., Ferguson, M. A., Knudsen, J. and Jones, T. A. (2001) Binding site differences revealed by crystal structures of *Plasmodium falciparum* and bovine acyl-CoA binding protein. *J. Mol. Biol.,* **309**(1), 181-192.

van Loon, L.C. and van Strien, E. A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.,* **55**(2), 85-97.

Vance, V. and Vaucheret, H. (2001) RNA silencing in plants - defense and counterdefense. *Science,* **292**(5525), 2277-2280.

Vogel, G. and Browse, J. (1996) Cholinephosphotransferase and diacylglycerol acyltransferase (substrate specificities at a key branch point in seed lipid metabolism). *Plant Physiol.,* **110**(3), 923-931.

Walz, C., Giavalisco, P., Schad, M., Juenger, M., Klose, J. and Kehr, J. (2004) Proteomics of curcurbit phloem exudate reveals a network of defence proteins. *Phytochemistry,* **65**(12), 1795-1804.

Wang, J.L., Wang, J. X. and Zhao, X. F. (2008) Molecular cloning and expression profiles of the acyl-CoA-binding protein gene from the cotton bollworm *Helicoverpa armigera*. *Arch. Insect Biochem. Physiol.,* **68**(2), 79-88.

Wanner, G. and Theimer, R. R. (1978) Membranous appendices of spherosomes (oleosomes). *Planta,* **140**(2), 163-169.

Waterhouse, P.M. and Helliwell, C. A. (2003) Exploring plant genomes by RNA-induced gene silencing. *Nat. Rev. Genet.,* **4**(1), 29-38.

Wedegaertner, P.B., Wilson, P. T. and Bourne, H. R. (1995) Lipid modifications of trimeric G proteins. *J. Biol. Chem.,* **270**(2), 503-506.

Weselake, R.J. (2005) *Storage production metabolism in microspore-derived cultures of Brassicaceae; Haploids in Crop Improvement II*, Berlin Heidelberg: Springer-Verlag.

Weselake, R.J. (2002) *Biochemistry and biotechnology of triacylglycerol accumulation in plants* (Anonymous) New York, Basel: Marcel Dekker, Inc.

Weselake, R.J. (2000) Lipid biosynthesis in cultures of oilseed rape. *In Vitro Cellular and Development Biology - Plant,* **36**(5), 338-348.

Weselake, R.J. and Taylor, D. C. (1999) The study of storage lipid biosynthesis using microspore-derived cultures of oil seed rape. *Prog. Lipid Res.,* **38**(5-6), 401-460.

Weselake, R.J., Wiehler, W. B., Patterson, N. A., Nykiforuk, C. L., Cianflone, K., Moloney, M. M. and Laroche, A. (2003) *Transformation of Brassica napus with cDNAs encoding proteins that stimulate in vitro triacylglycerol biosynthesis; Plant Biotechnology 2002 and Beyond* (I.K. Vasil, ed) Netherland: Kluwer Academic Publishers.

Wilkinson, J.E., Twell, D. and Lindsey, K. (1997) Activities of CaMV 35S and nos promoters in pollen: Implications for field release of transgenic plants. *J. Exp. Bot.,* **48**(2), 265-275.

Xiao, S., Gao, W., Chen, Q. F., Ramalingam, S. and Chye, M. L. (2008a) Overexpression of membrane-associated acyl-CoA-binding protein ACBP1 enhances lead tolerance in *Arabidopsis*. *Plant J.,* **54**(1), 141-151.

Xiao, S., Li, H. Y., Zhang, J. P., Chan, S. W. and Chye, M. L. (2008b) *Arabidopsis* acyl-CoA-binding proteins ACBP4 and ACBP5 are subcellularly localized to the cytosol and ACBP4 depletion affects membrane lipid composition. *Plant Mol. Biol.,***68**, 571-583.

Xiao, S., Chen, Q. F., Chye, M. L. (2009) Light-regulated Arabidopsis ACBP4 and ACBP5 encode cytosolic acyl-CoA-binding proteins that bind phophatidylcholine and oleyol-CoA esters. *Plant Physiol Biochem*., **47**(10), 926-933.

Xiao, S. and Chye, M. (2009) An *Arabidopsis* family of six acyl-CoA-binding proteins has three cytosolic members. *Plant Physiology and Biochemistry,* **47**(6), 479-484.

Yurchenko, O., Weselake, R. J., Nykiforuk, C. L. and Mononey, M. M. (2008) Transgenic expression of acyl-CoA-binding proteins in plants. International patent PCT/CA2008/000500.

Zeng, B., Cai, X. and Zhu, G. (2006) Functional characterization of a fatty acyl-CoA-binding protein (ACBP) from the apicomplexan *Cryptosporidium parvum*. *Microbiology,* **152**(8), 2355-2363.

Zhang, M., Xu, S., Chye, M. and Liang, C. (1999) Identification of a rice cDNA encoding the acyl-CoA-binding protein (ACBP). *Acta Phytophysiologica Sinica,* **25**(4), 327.

Zheng, B.S., Ronnberg, E., Viitanen, L., Salminen, T. A., Lundgren, K., Moritz, T. and Edqvist, J. (2008) *Arabidopsis* sterol carrier protein-2 is required for normal development of seeds and seedlings. *J. Exp. Bot.,* **59**(12), 3485-3499.

Zheng, Z. and Zou, J. (2001) The initial step of the glycerolipid pathway. Identification of glycerol-3-phosphate/dihydrozyacetone phosphate dual substrate acyltransferases in *Saccharomyces cerevisiae*. *J. Biol. Chem.,* **276**(45), 41710-41716.

Zheng, Z., Xia, Q., Dauk, M., Shen, W., Selvaraj, G. and Zou, J. (2003) *Arabidopsis* AtGPAT1, a member of the membrane-bound glycerol-3-phosphate acyltransferase gene family, is essential for tapetum differentiation and male fertility. *Plant Cell,* **15**(8), 1872-1887.

Zimmerman, A.W. and Veerkamp, J. H. (2002) New insights into the structure and function of fatty acid-binding proteins. *Cell Mol. Life Sci.,* **59**(7), 1096-1116.

Appendix 1. Molecular cloning procedures

A1.1. Materials used for molecular cloning

The restriction endonucleases were purchased from New England Biolabs Inc. (Mississauga, ON, Canada), Invitrogen Life Technologies (Burlington, ON, Canada) or Amersham Biosciences (Baie d'Ufré, QC, Canada). DNA-modifying enzymes: Taq DNA polymerase, T4 DNA ligase and calf intestine alkaline phsphatase (CIAP) were purchased from New England Biolabs (Mississauga, ON, Canada); Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA, USA).

Oligonucleotides were synthesized by Invitrogen Life Technologies and Integrated DNA Technologies, Inc. (Coralville, IA, USA). Oligonucleotide sequences are presented in Table A1.1. Some oligonucleotides contained restriction endonuclease sites that were incorporated into PCR products and used in cloning procedures.

A1.2. Molecular cloning techniques

Recombinant DNA techniques such as restriction endonuclease digestion and ligation were performed as described by Sambrook et al. (1989). Plasmid preparation and DNA gel extraction were performed using QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit, respectively, following the manufacture's protocols (Qiagen, Mississauga, ON, Canada).

Plymerase chain reaction (PCR) was performed using 200 μM of each dNTP, 0.1 volume of PCR reaction buffer (for each respective DNA polymerase), 1 μM of forward and reverse primer oligonucleotides each, 0.5 – 1 unit of Taq or

Pfu DNA polymerase and 10-500 ng of DNA template to a final reaction volume of 25 µL. The general PCR thermal cycling condition were: 2 min preheat at 94°C followed by 30 cycles of DNA denaturation at 94°C for 1 min, 30 seconds of annealing at 50 to 58°C and extension at 72°C for 1 min. After the final cycle, the PCR samples were incubated for 5 min at 72°C for complete extension and cooled to 4°C until used for analysis.

A1.3. Bacterial transformation

A1.3.1 *Escherichia coli* transformation

Escherichia coli competent DH5α were prepared using the standard heat shock protocol (Sambrook *et al.*, 1989). Competent cells (100 µL) were mixed with 0.1 µg of plasmid DNA and incubated on ice for 30 minutes. Then cells were heat-shocked for 45 sec. at 42°C followed by 2 minute incubation on ice and, after addition of 500 µL LB broth, were incubated on shaker at 37°C for 1 hour. After a final incubation, several aliquots (10, 50, 100 µL) were plated on the LB plates containing the appropriate antibiotic.

A1.3.2. *Agrobacterium tumefaciens* transformation

Agrobacterium tumefaciens cells (40 µL) were mixed with 0.4 µg of plasmid DNA and incubated on ice for 5 minutes. After incubation, the mixture was placed in a cold 0.2 cm electroporation cuvette and electroporated with the pulse generator (Biorad Laboratories Ltd.) set at 25 µF, 2.5 kV and 200 Ω. Immediately after electroporation, cells were suspended in 500 µL of LB broth and incubated at 28°C for 2 hours. Small aliquots (2, 5, 10 µL) were plated onto LB plates containing appropriate antibiotics.

Table A1.1. Sequences of the oligonucleotides used in the study.

Name	Oligo Sequence (5' to 3' orientation)	Description
385	ATGCCCAAATCTCCATGCAT	*phaP* forward primer
1109	CCATAAGCCGTCACGATTC	*phaT* reverse primer
1456	GGTAGTGTGCTGGCCA	*AthOleosin* reverse primer
2035	TCATGATAGACATTGTGATGACACAGTC	*D9 scFv* forward primer with *BspHI* site
2036	ATTTAAATTGGTTGACTCGAGACTG	*D9 scFv* reverse primer with *SwaI, XhoI* sites
2037	CACACTACTAGTTCAGTCATGAACTGATCA CAAGCTTACCCTATG	forward oligo adaptor with *BspHI, SpeI* sites
2038	CATAGGGTAAGCTTGTGATCAGTTCATGAC TGAACTAGTAGTGTG	reverse oligo adaptor with *BspHI, SpeI* sites
2061	CCATGGGTTTGAAGGAGGAC	*BnACBP* forward primer with *NcoI* site
2062	CCATGGCTGAAGCGGAGGAAG	*BnACBP* reverse primer with *NcoI* site
2082	CTCATGATGTGCAGAAGCTTAAC	*BBE* motif (B82) forward primer with *BspHI* site
2083	TCATGACAATAAACGACGGTTTC	*BBE* motif (B82) reverse primer with *BspHI* site
2422	GGATCCATGGGTTTGAAGGAGGAC	*BnACBP* forward primer with *BamHI* site
2423	CTCGAGTCAAGCTGAAGCGGA	*BnACBP* reverse primer with *XhoI* site
MT-B1	TAAGAGCACTTAATAGATCAC	*BnACBP1* variant reverse primer
MT-B2	TCATCAAGTTGAAGCGGCGGAAGCCTCTG	*BnACBP2* variant reverse primer
MT-B3	ATATAAATTCATCAAGTTGAAGC	*BnACBP3* variant reverse primer
MT-F1	TCTGACGTCTAATCATCATG	*BnACBP1, 2, 3* variants forward primer
OY-17	CACCATGGGTTTGAAGGAGGAATTTG	*AthACBP* forward primer with CACC sequence for TOPO cloning
OY-18	CAGGTTGAAGCCTTGGAAGCAGC	*AthACBP* reverse primer
OY-27	ATTACTCGAGCCATGGGTTTGAAGG	*AthACBP*(dir.) forward primer with *XhoI* site
OY-28	TAATGAATTCGGTTGAAGCCTTGG	*AthACBP*(dir.) reverse primer with *EcoRI* site
OY-29	ATTATCTAGACCATGGGTTTGAAGGAGG	*AthACBP*(inv.) forward primer with *XbaI* site
OY-30	TAATAAGCTTGGTTGAAGCCTTGG	*AthACBP*(inv.) reverse primer with *HindIII* site
OY-31	AATCATGACTGAAGCGGAGGAAGCC	*BnACBP* reverse primer with *BspHI* site
OY-32	TTTCATGACGCACAATCCCACTATCC	*GFP* forward primer with *BspHI* site
OY-33	TTTCATGAGTTTGTATAGTTCATCCATGCC	GFP reverse primer with *BspHI* site
PP-F	GATGCAATCTCTCATTCCGATAGTC	*AthPP2A* forward primer
PP-R	CAAGCATGGCCGTATCATGT	*AthPP2A* reverse primer
PP-VIC	ACCAAGCGGTTGTGGAG	TaqMan MGB probe for *AthPP2A*

A1.4. Molecular cloning of *BnACBP* genetic constructs

A1.4.1. Cloning of *Oleo-ACBP, ACBP-Oleo, OleoH3P-ACBP and B82-Oleo-ACBP* constructs

A 286bp cDNA encoding BnACBP was cut out of the pBlueScriptS/K+ACBP plasmid with *NcoI* and *HindIII* and ligated into the pSBS4008 plasmid containing oleosin, resulting in a translational fusion (Oleo-ACBP), designated pSBS4140 (Figure A1.1a).

A PCR fragment encoding BnACBP cDNA was amplified from pBlueScriptS/K+ACBP using primers 2061 and 2062, cut with *NcoI*, and ligated into *NcoI* site of pSBS4007 vector. The ligation product gave an in-frame fusion of BnACBP cDNA at the 5' end of Oleosin gene (ACBP-Oleo construct), resulting in a pSBS4141 vector (Figure A1.1b).

B82 DNA fragment, encoding 82 residues of the BBE, was amplified from pB82P1 using 2082 and 2083 primers, which introduced *BspHI* sights at the 5' and 3' ends of the PCR product. B82 was cut with *BspHI* and ligated into *NcoI* site of pSBS4007 vector. The resulting plasmid was cut with *PstI* and *SphI*, and DNA fragments PhaP- ½B82 (phaseolin promoter and first 178 bp of B82) and ½B82- ½Oleo (last 59 bp of B82 and first 404 bp of At Oleosin gene) were purified. *PstI-SphI* fragment of pSBS4140 including PhaP and first 404 bp of At Oleosin gene was substituted with PhaP- ½B82 fragment. The resulting plasmid was cut with *SphI* and ligated with ½B82- ½Oleo fragment. The final ligation product represents in-frame fusion of B82, oleosin gene and BnACBP (B82-Oleo-ACBP construct), and resulted in pSBS4142vector (Figure A1.1c).

OleoH3P (modified gene encoding oleosin with 32 amino acids deletion from the hydrophobic domain) fragment was cut of the pOLG-H3P plasmid with *NcoI* and *HindIII*, and ligated with pSBS4006 pre-treated with the same restriction enzymes. The adaptor (annealed oligonucleotides 2037 and 2038) was inserted into pSBS4006-OleoH3P cut with *SpeI* and *HindIII* in order to introduce *BspHI* site. The resulting plasmid was cut with *BspHI* and *HindIII* and ligated with *NcoI-HindIII* fragment of pBlueScriptS/K+ACBP, encoding BnACBP. The ligation product was a pSBS4143 vector carrying a translational fusion of OleoH3P and BnACBP (Figure A1.1d).

A1.4.2. Cloning of *ACBP* and *D9-ACBP* constructs

Two expression cassettes were made for cytosolic expression of acyl-CoA binding proteins: BnACBP alone and BnACBP fusion with singe-chain fragment of the monoclonal antibody against *A. thaliana* 18 kDa oleosin (D9).

NcoI-HindIII fragment encoding ACBP was cloned into corresponding sites on pSBS4006 resulting in pSBS4146 vector with ACBP expression construct (Figure A1.1e).

DNA fragment encoding for D9 was amplified from pSBS4415 with primers 2035 and 2036, cut with *SwaI* and *BspHI*, and ligated with pSBS4006 cut with *SwaI* and *NcoI*. The resulting plasmid was cut with *XhoI* and *HindIII* and ligated with ACBP fragment with compatible 3' and 5' ends. The ligation product represented and in-frame fusion between D9 and ACBP (D9-ACBP construct) on a pSBS4147 vector (Figure A1.1f).

A1.4.3. Cloning of *ACBP-KDEL* and *D9-ACBP-KDEL* constructs

Two control constructs for the luminal targeting of ACBP were used in transformation program: ACBP-KDEL and D9-ACBP-KDEL.

ACBP-KDEL expression cassette was constructed by inserting *HindIII-NcoI* fragment encoding ACBP-KDEL from pBlueScriptS/K+ACBP-KDEL into pSBS4011 vector cut with *NcoI* and *HindIII*. The resulting vector was called pSBS4144 (Figure A1.1g).

DNA fragment encoding for D9 was amplified from pSBS4415 with primers 2035 and 2036, and cut with *XhoI* and *BspHI*. DNA fragment encoding ACBP-KDEL was cut of pBlueScriptS/K+ACBP-KDEL with *HindIII* and *XhoI*. Both DNA fragments were ligated with pSBS4011 vector cut with *NcoI* and *HindIII*, yielding pSBS4145 vector containing D9-ACBP-KDEL construct (Figure A1.1h).

A1.5. Construction of the binary vectors for *B. napus* transformation with *BnACBP* constructs.

Molecular cloning of the constructs designed for expression in *B. napus* developing seeds was performed by cloning ACBP-Oleo, OleoH3P-ACBP and ACBP expression cassettes including phaseolin promoter and terminator into the *EcoRI* site of the pGreen0029 plasmid. The resulting vectors were named pGreen0029-41, pGreen0029-43 and pGreen0029-46, respectively (Figure A1.2).

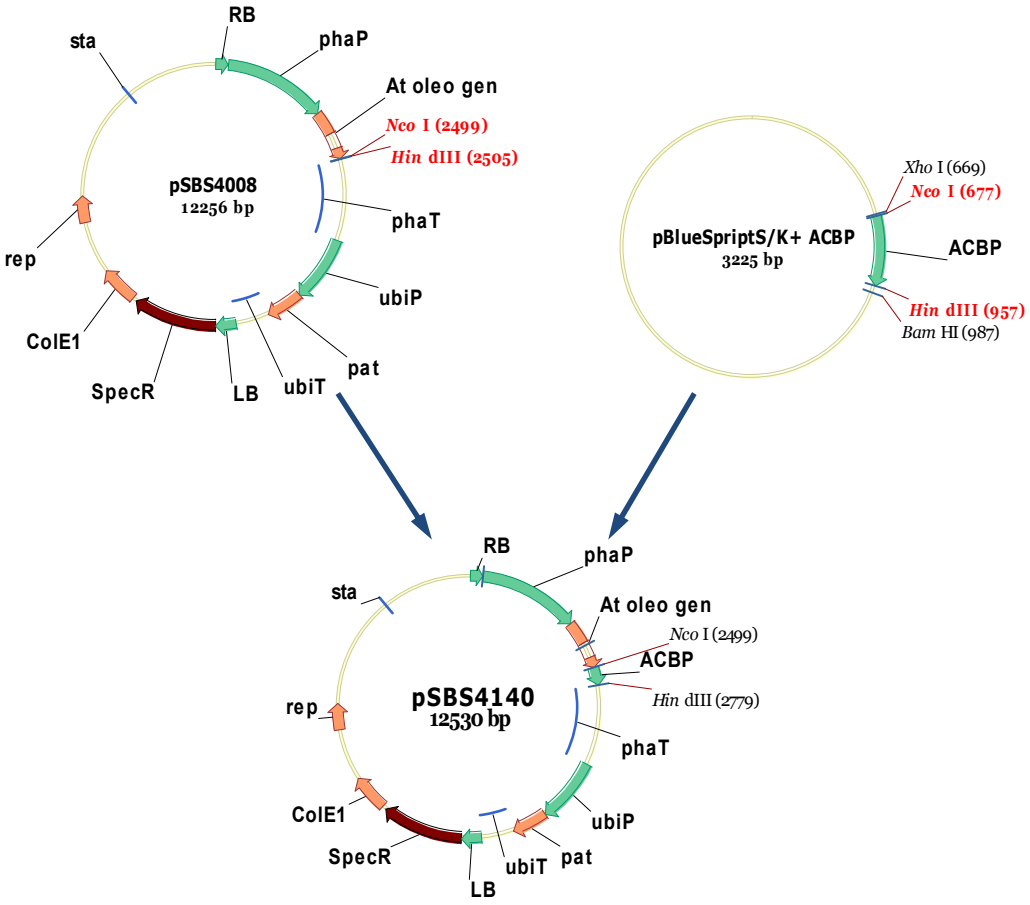


Figure A1.1a. Schematic drawing of the cloning of *Oleo-ACBP* construct (pSBS4140 vector) for *A. thaliana* transformation.

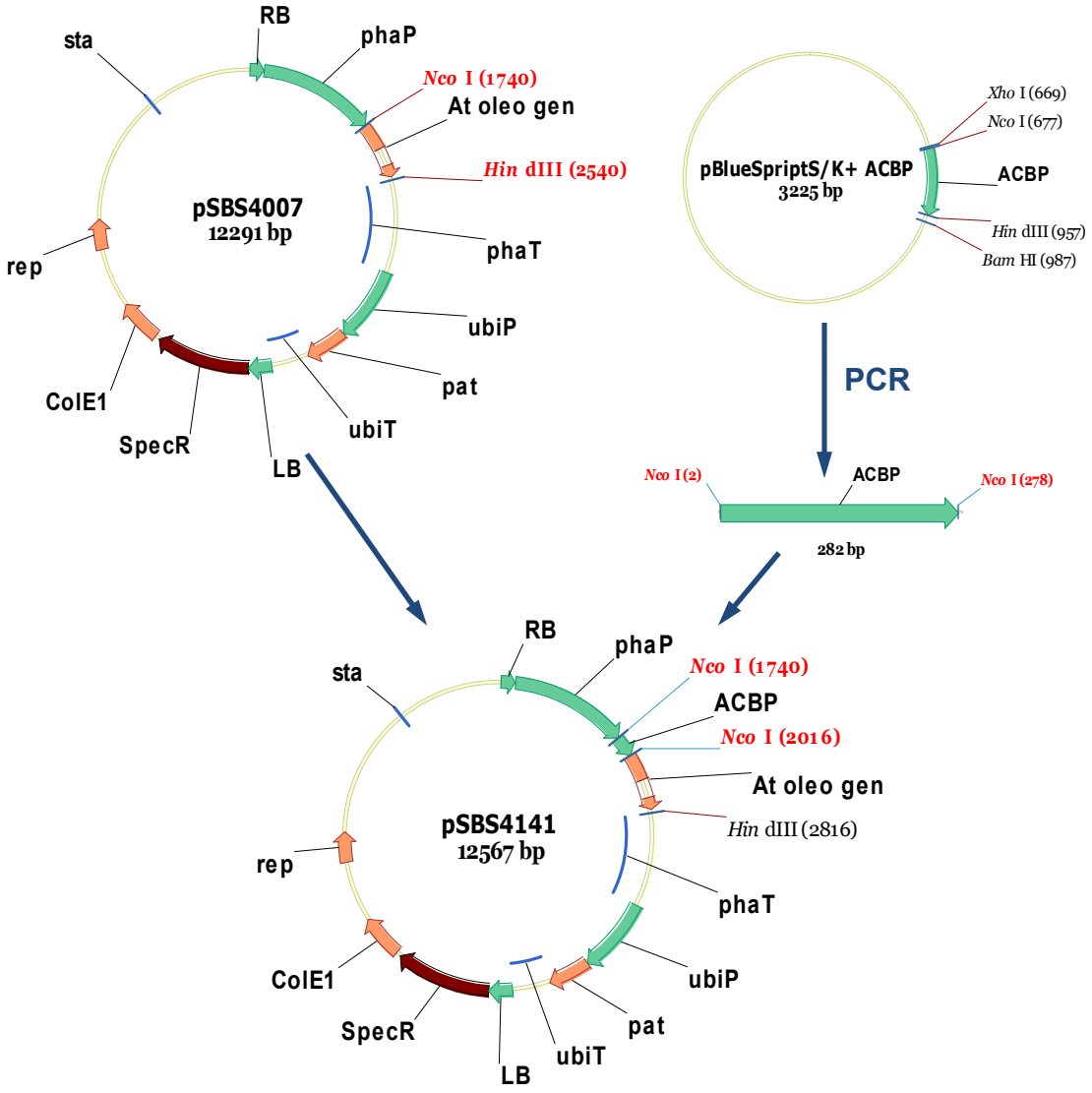


Figure A1.1b. Schematic drawing of the cloning of *ACBP-Oleo* construct (pSBS4141 vector) for *A. thaliana* transformation.

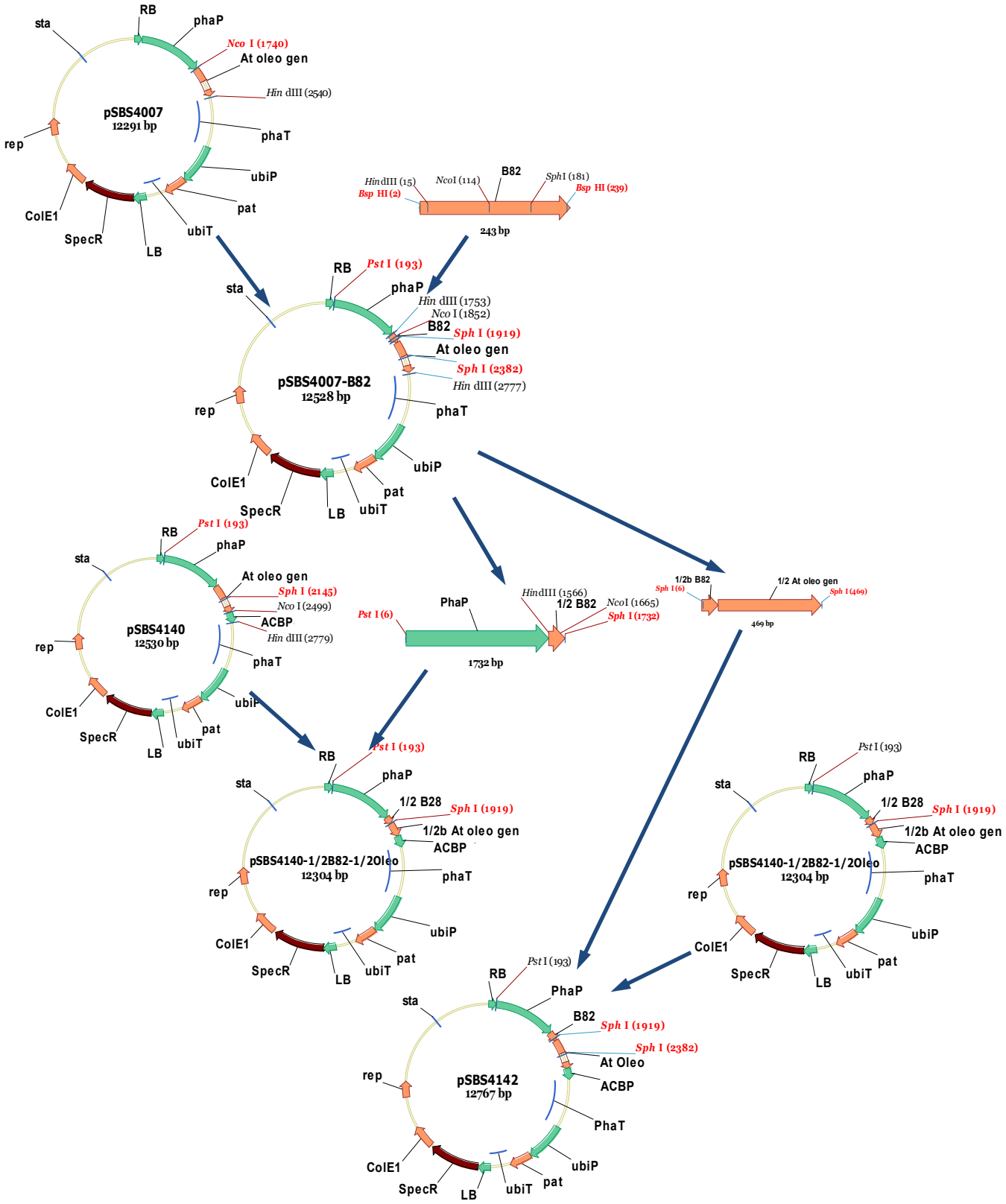


Figure A1.1c. Schematic drawing of the cloning *B82-Oleo-ACBP* construct (pSBS4142 vertor) for *A. thaliana* transformation.

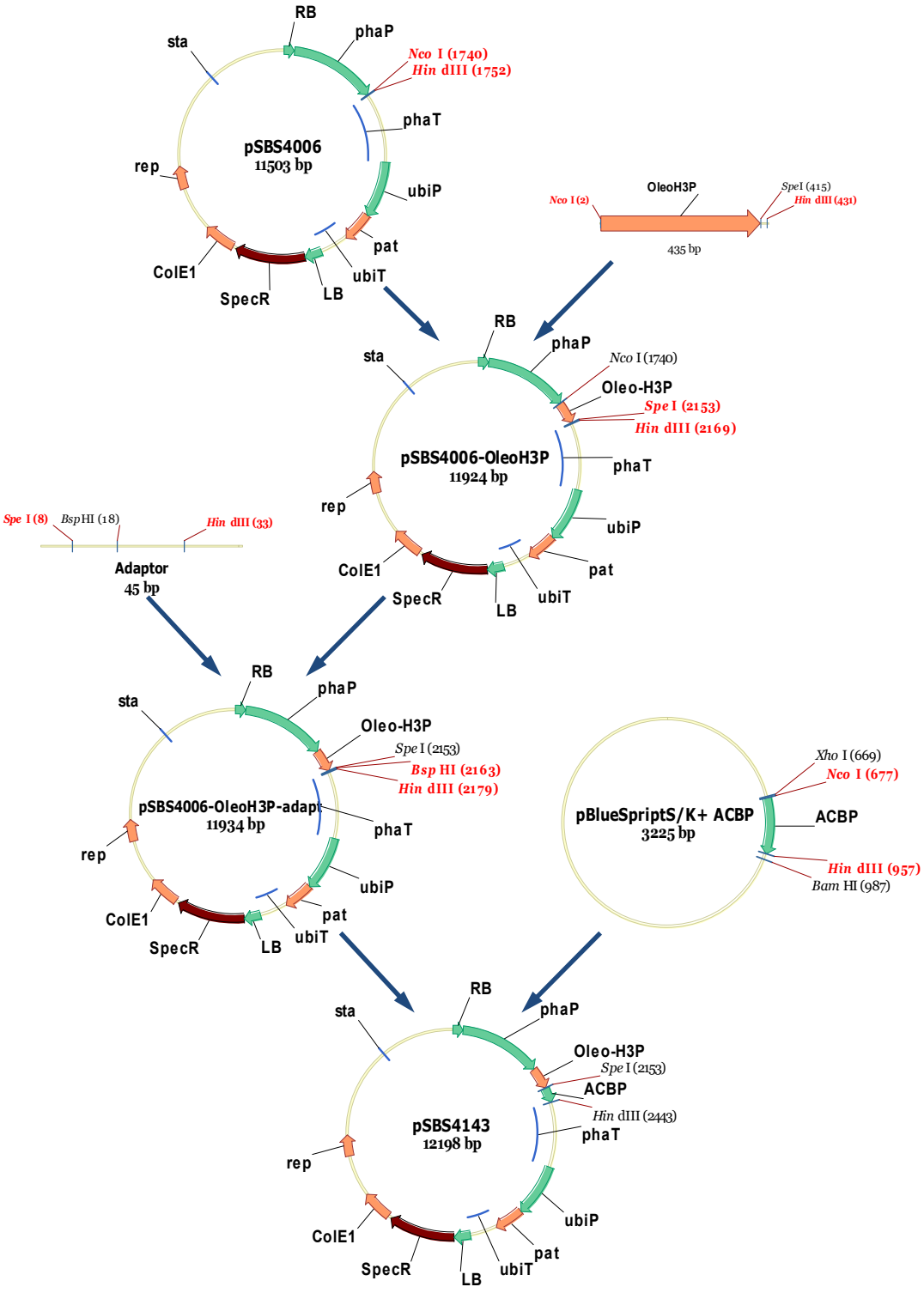


Figure A1.1d. Schematic drawing of the cloning of *OleoH3P-ACBP* construct (pSBS4143 vector) for *A. thaliana* transformation.

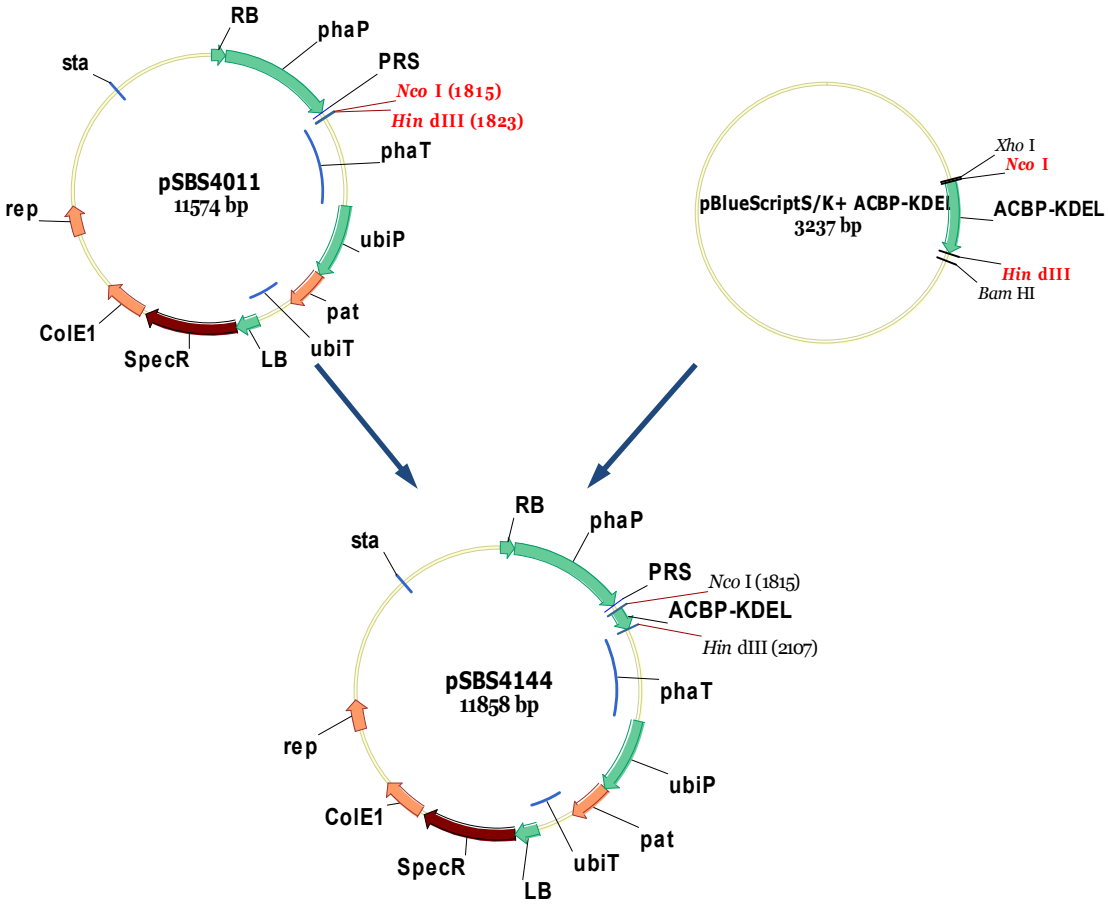


Figure A1.1e. Schematic drawing of the cloning of *ACBP-KDEL* construct (pSBS4144 vector) for *A. thaliana* transformation.

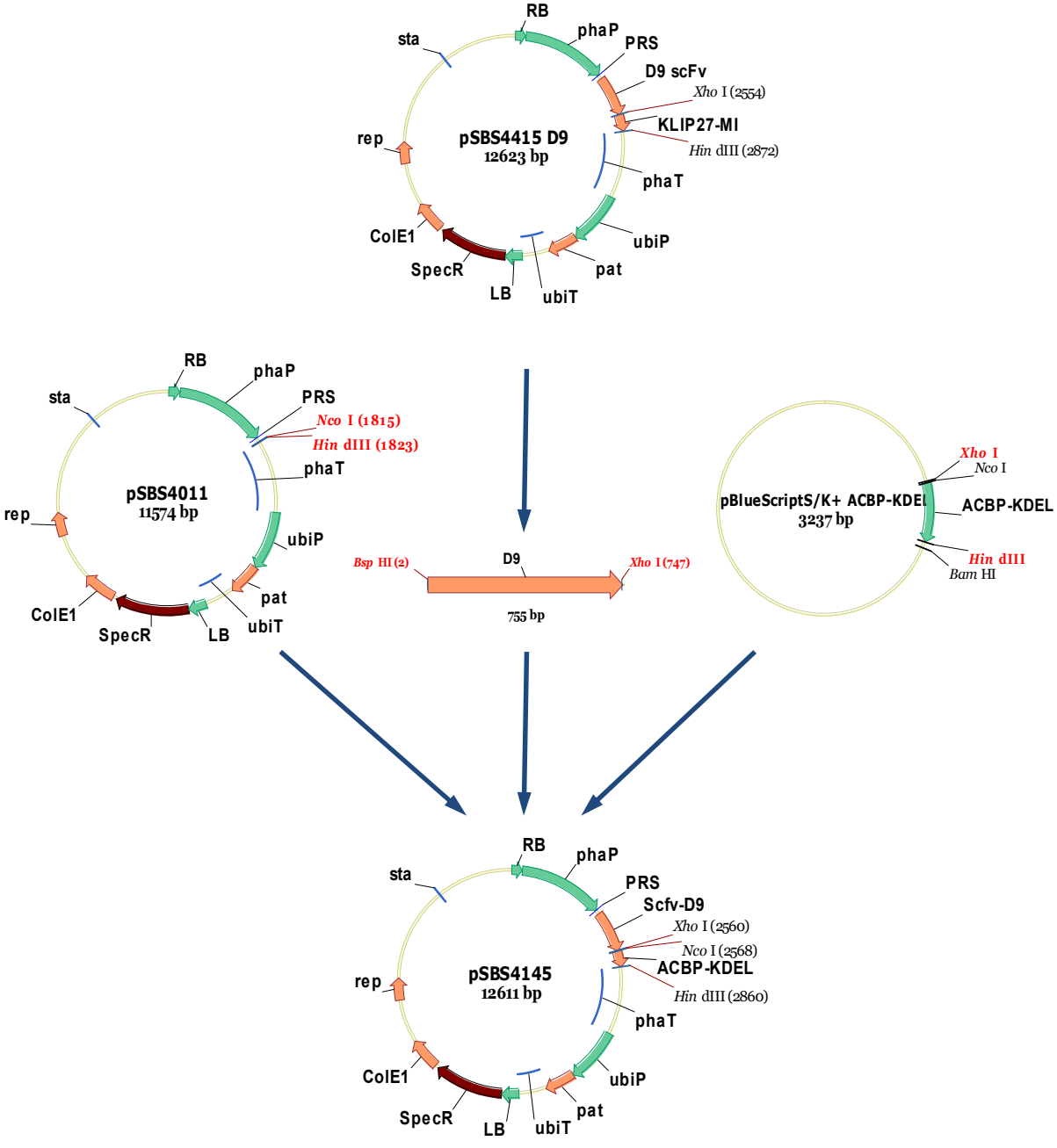


Figure A1.1f. Schematic drawing of the cloning of *D9-ACBP-KDEL* contruct (pSBS4145 vector) for *A. thaliana* transformation.

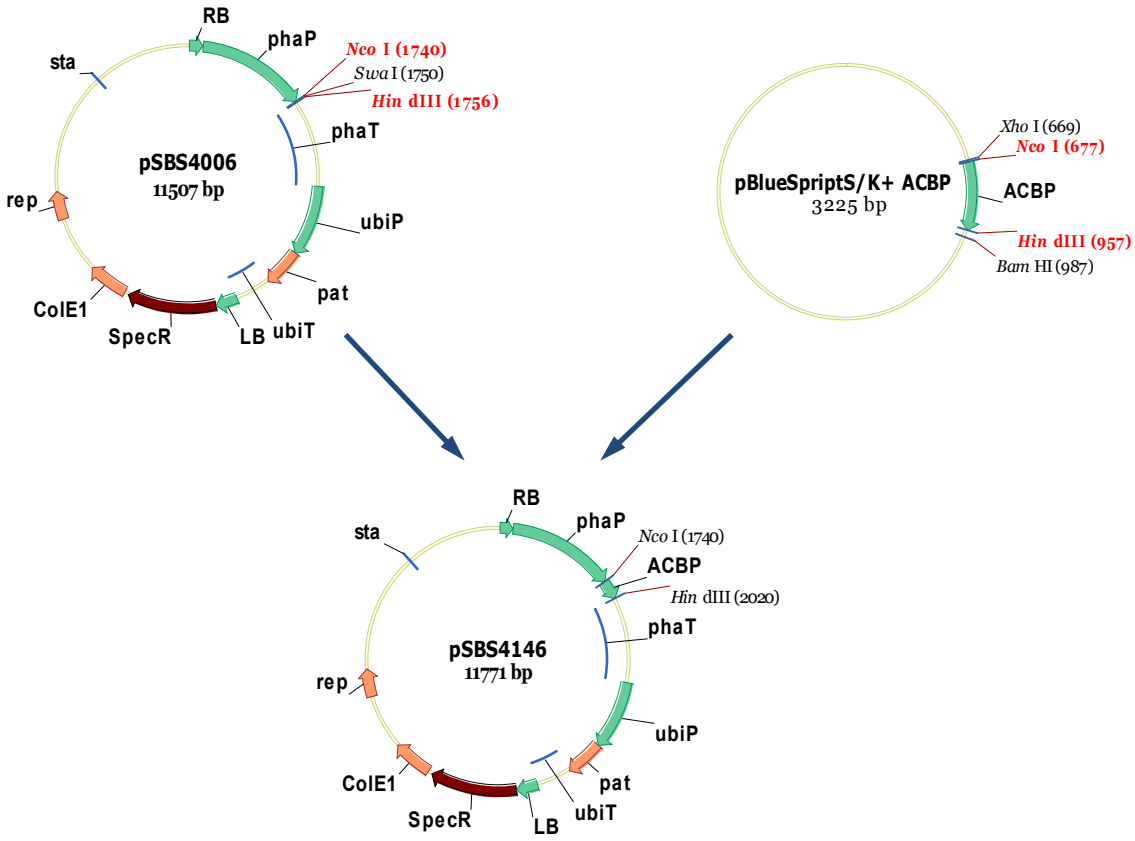


Figure A1.1g. Schematic drawing of the cloning of *ACBP* construct (pSBS4146 vector) for *A. thaliana* transformation.

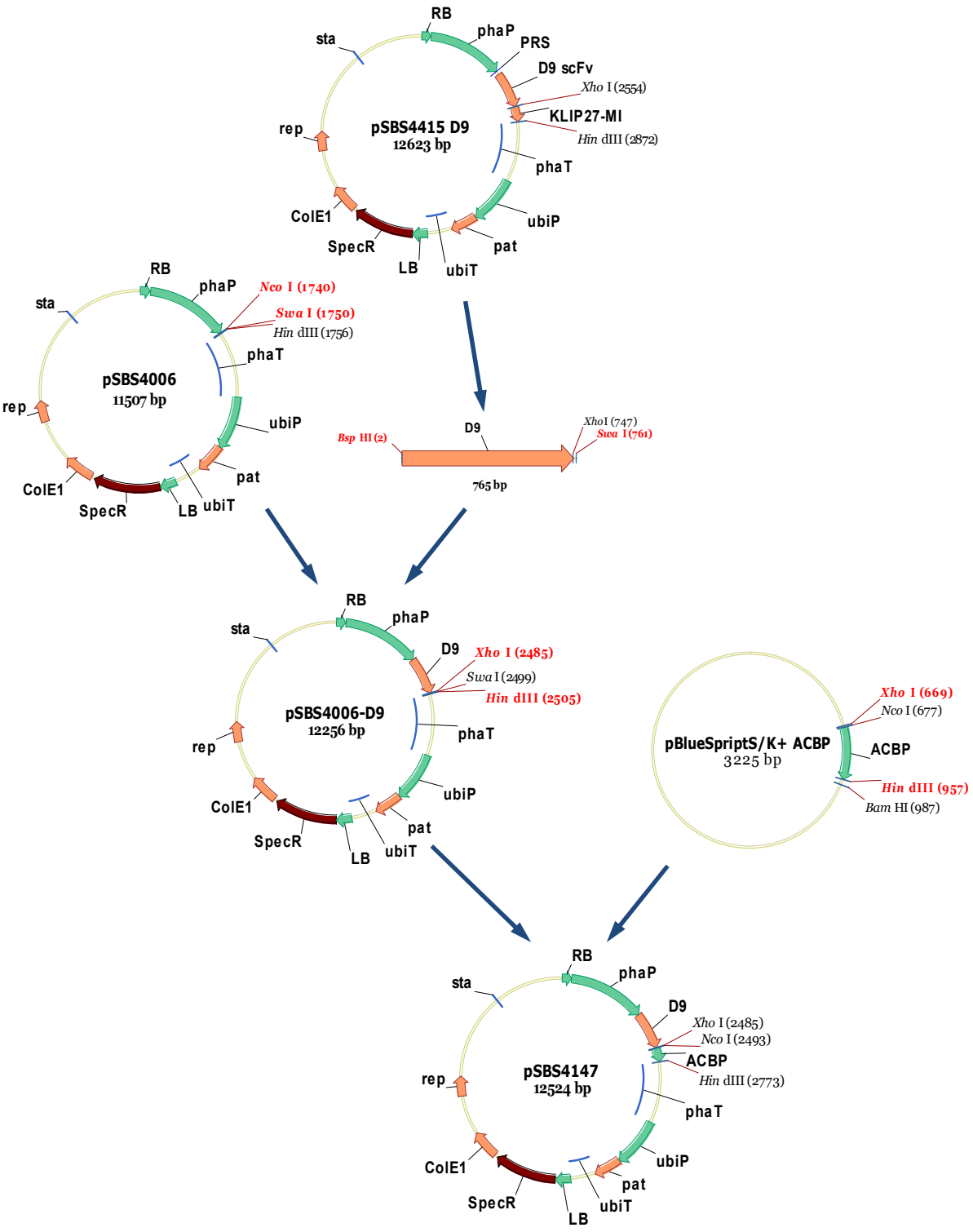


Figure A1.1h. Schematic drawing of the cloning strategy of *D9-ACBP* construct (pSBS4147 vector) for *A. thaliana* transformation.

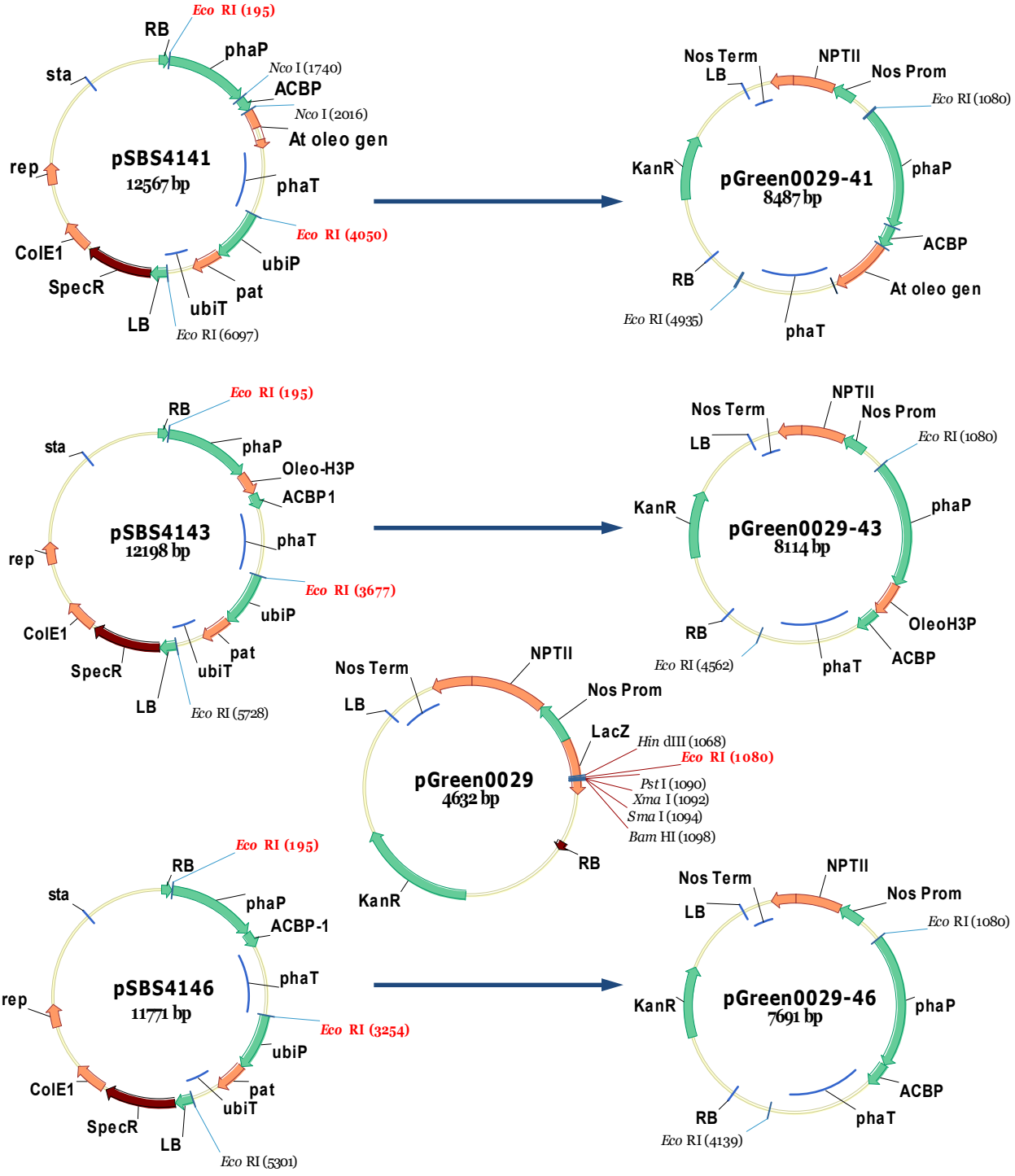


Figure A1.2. Schematic drawing of the cloning of *BnACBP* molecular constructs for *B. napus* transformation (pGreen0029-41, pGreen0029-43 and pGreen0029-46 vectors).

A1.6. Molecular cloning of *GFP-BnACBP* constructs

DNA fragment encoding GFP was amplified from p3355-GFP plasmid with OY-32 and OY-33 primers, cut with *BspHI*, and ligated into *NcoI* site of pSBS4007, pSBS4007-ACBP, pSBS4143, pSBS4144, and pSBS4146 to produce 5' in-frame fusion of GFP with Oleosin, ACBP-Oleosin, OleoH3P-ACBP, ACBP-KDEL and ACBP constructs (resulting vectors pSBS4007-GFP, pSBS4141-GFP, pSBS4143-GFP, pSBS4144-GFP, and pSBS4146-GFP, respectively) (Figure A1.3). DNA fragment encoding ACBP amplified from pSBS4141 with OY-31 and 2062, cut with *NcoI* and *BspHI*, and inserted into *NcoI* site of pSBS4007 in order to obtain pSBS4007-ACBP, which has singe *NcoI* site opposite to pSBS4141, which has two *NcoI* sites.

A1.7. Molecular cloning of *AthACBP* hairpin constructs

A1.7.1. Cloning of *AthACBP_RNAi* constructs for constitutive expression

cDNA encoding *A. thaliana* 10 kDa ACBP was amplified from *A. thaliana* cDNA using primers OY-17 and OY-18 (Table A1.1). *AthACBP* was directionally TOPO-cloned into pENTR/D-TOPO cloning vector (Invitrogen, Burlington, ON). Gateway cloning of *AthACBP* hairpin construct was accomplished by LR reaction between pENTR/D-TOPO-AthACBP and destination vectors pWatergate or pHellsgate12 (CSIRO Plant Industry, Townsville, Australia), designed for a constitutive RNAi expression from *Arabidopsis* Rubisco small subunit promoter (ARbcP) and CaMV35S promoter, respectively. Molecular cloning of the final binary vectors pSBS4148 and pSBS4149, which resulted from Gateway cloning

of AthACBP into pWatergate and pHellsgate12, respectively, is illustrated in Figure A1.4.

A1.7.2. Cloning of *AthACBP_RNAi* constructs for seed-specific expression

RNAi construct for seed specific silencing of AthACBP was made using conventional cloning methods. The direct sequence of AthACBP (AthACBPdir) was amplified with OY-27 and OY-28 primers from pENTR/D-TOPO-AthACBP to introduce *XhoI* site at 5' and *EcoRI* site at 3' end. Inverted sequence of AthACBP (AthACBPinv) was amplified with OY-29 and OY-30 primers to introduce *XbaI* and *HindIII* sites at 5' and 3' ends, respectively. Direct and inverted *AthACBP* were inserted into *XhoI- EcoRI* and *XbaI-HindIII* sites of pHannibal cloning vector (CSIRO Plant Industry, Townsville, Australia), respectively, resulting in pHannibal-AthACBP_RNAi. AthACBP_RNAi cassette was cut off the pHannibal backbone and inserted into *XhoI-XbaI* sites of pGreen0229 binary vector (based on pGreen0029, John Innes Centre, Norwich, UK), resulting in pGreen-AthACBP_RNAi (Figure A1.5).

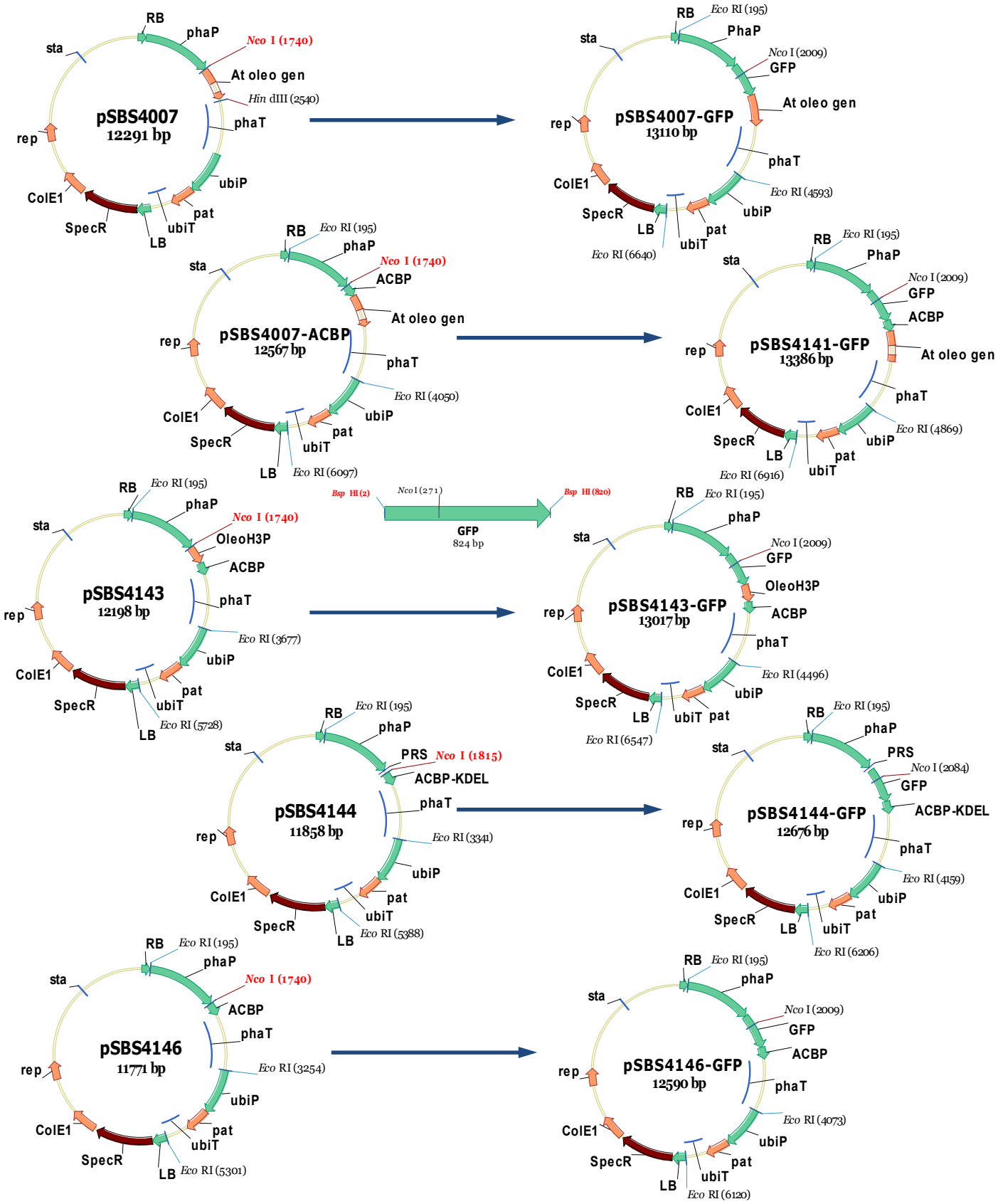


Figure A1.3. Schematic drawing of the cloning of *GFP* molecular constructs for *A. thaliana* transformation (pSBS4007-GFP, pSBS4141-GFP, pSBS4143-GFP, pSBS4144-GFP, and pSBS4146-GFP vectors).

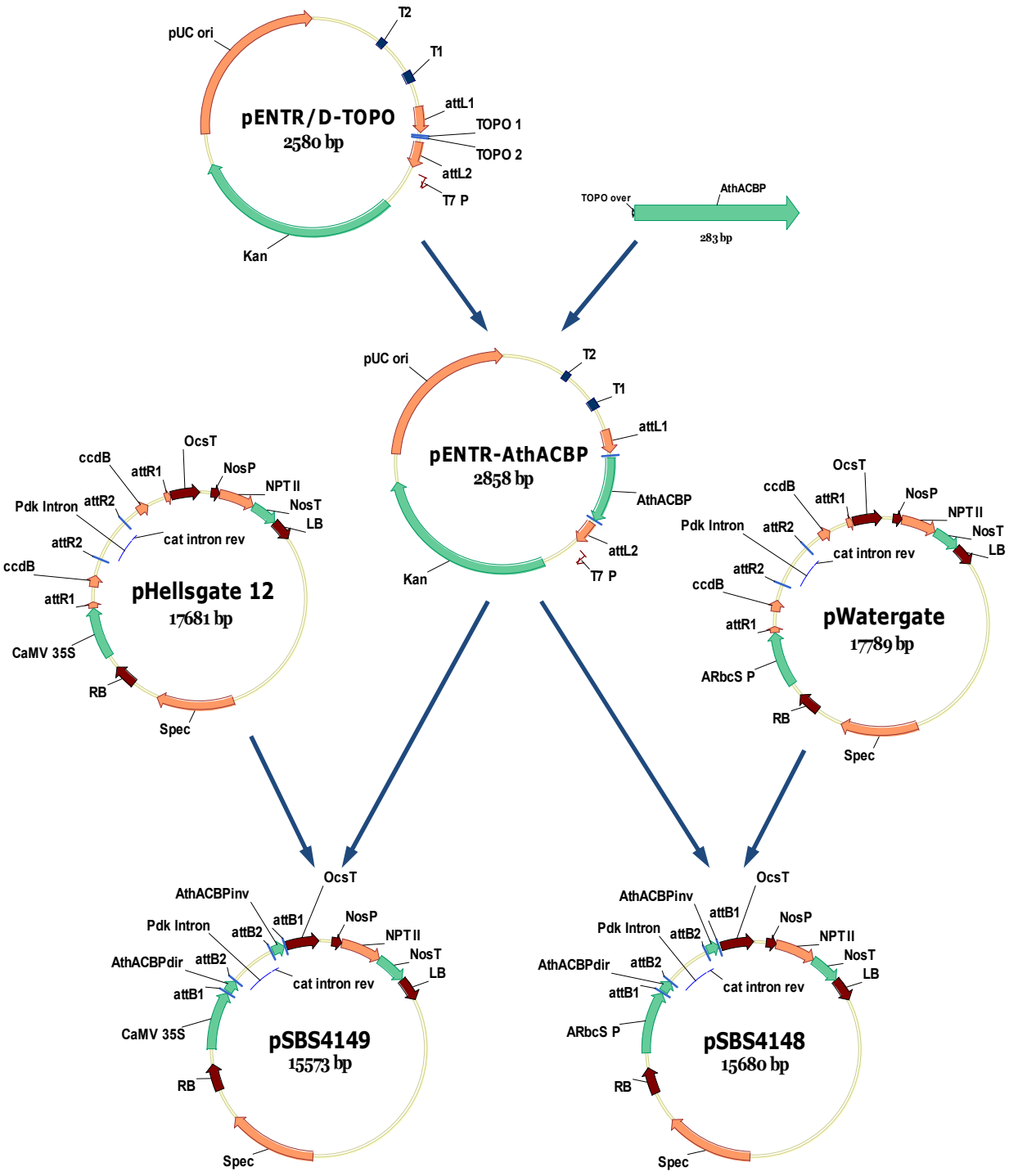


Figure A1.4. Schematic drawing of the molecular cloning of pSBS4148 and pSBS4149 vectors used for *A. thaliana* transformation with constitutively expressed *AthACBP_RNAi* constructs.

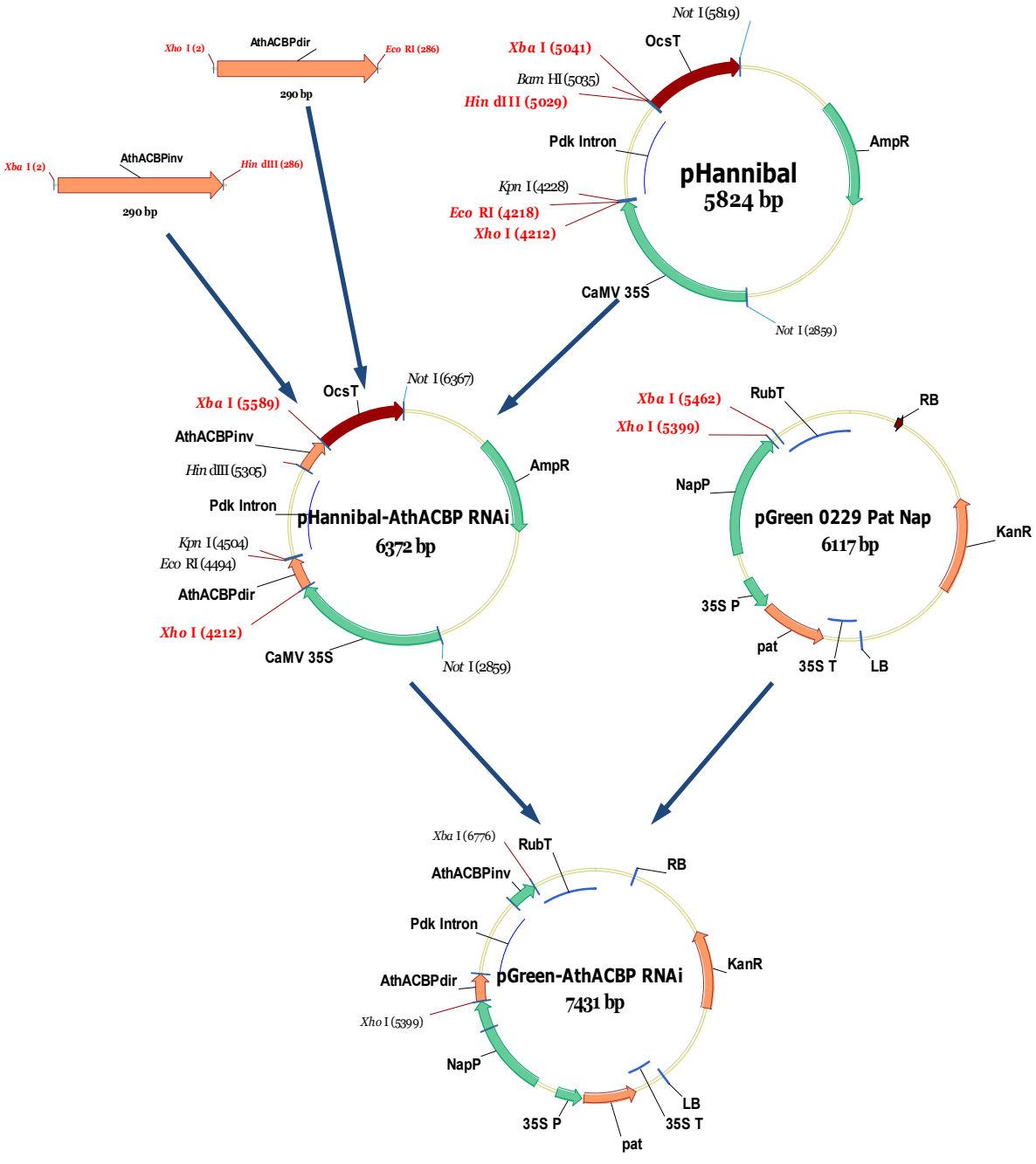


Figure A1.5. Schematic drawing of the molecular cloning of pGreen-AthACBP_RNAi vector used for *A. thaliana* transformation with *AthACBP_RNAi* construct for seed-specific expression.

Appendix 2. Analysis of the fatty acid composition (mol%) of seed oil

Table A2.1. FA composition of the seed oil from *A. thaliana* T_2 seeds transformed with *BnACBP* constructs. Four T_2 lines with the highest PUFA% in seed oil within each construct were included in the analysis. Mean mol% ± SD of the means (n=4). ($^{▲}$/$^{▼}$) values significantly greater/ smaller than WT at α=0.05; (^{Δ}/◊) values significantly greater/ smaller than WT at α=0.1.

Construct	16:0	18:0	18:1	18:2	18:3	20:1
WT	8.25 ± 0.06	3.69 ± 0.04	15.75 ± 0.06	26.79 ± 0.08	17.04 ± 0.14	19.46 ± 0.04
Null Vector	8.64 ± 0.21	3.32 ± 0.72	17.04 ± 0.26	27.44 ± 2.16	16.34 ± 0.73	19.56 ± 0.75
ACBP-Oleo	9.04 ± 0.44^{Δ}	2.47 ± 0.18$^{▼}$	15.44 ± 1.06	33.08 ± 1.16$^{▲}$	16.08 ± 0.92	16.47 ± 0.80$^{▼}$
OleoH3P-ACBP	8.67 ± 0.28	2.53 ± 0.14$^{▼}$	16.84 ±1.28	32.01 ± 0.22$^{▲}$	16.13 ± 1.08	16.72 ± 0.27$^{▼}$
B82-Oleo-ACBP	10.55 ± 0.96$^{▲}$	2.22 ± 0.26$^{▼}$	15.12 ± 1.02	35.88 ± 1.96$^{▲}$	15.41 ± 1.34$^{▼}$	14.94 ± 1.82$^{▼}$
ACBP-KDEL	8.88 ± 0.37	2.66 ± 0.18$^{▼}$	16.23 ± 0.86	34.04 ± 0.71$^{▲}$	13.25 ± 0.19$^{▼}$	18.11 ± 0.53◊
D9-ACBP-KDEL	9.21 ± 0.61$^{▲}$	2.48 ± 0.09$^{▼}$	18.41 ± 0.99$^{▲}$	32.70 ± 0.56$^{▲}$	12.47 ± 0.27$^{▼}$	17.51 ± 0.76$^{▼}$
ACBP	7.41 ± 0.39◊	2.58 ± 0.25$^{▼}$	16.47 ± 0.37	30.33 ± 0.74$^{▲}$	19.54 ± 1.36$^{▲}$	16.29 ± 0.51$^{▼}$
D9-ACBP	8.51 ± 0.11	2.42 ± 0.11$^{▼}$	17.06 ± 1.08	31.62 ± 0.54$^{▲}$	14.85 ± 0.54$^{▼}$	18.35 ± 0.31◊

Table A2.2. Composition of FA classes in *A. thaliana* T_2 seeds transformed with *BnACBP* constructs. Four lines with the highest PUFA% in seed oil within each construct are included in the analysis. Mean mol% ± SD of the means (n=4). ($^{▲}$/$^{▼}$) values significantly greater/ smaller than WT at α=0.05.

Construct	SFA	MUFA	PUFA
WT	14.11 ± 0.11	39.51 ± 0.09	46.37 ± 0.20
Null Vector	14.04 ± 1.40	40.87 ± 0.72	45.07 ± 1.58
ACBP-Oleo	13.30 ± 0.48	36.21 ± 1.17$^{▼}$	50.49 ± 0.84$^{▲}$
OleoH3P-ACBP	12.71 ± 0.51	37.94 ± 1.48	49.34 ± 0.97$^{▲}$
B82-Oleo-ACBP	14.00 ± 0.96	33.78 ± 1.87$^{▼}$	52.20 ± 1.08$^{▲}$
ACBP-KDEL	12.95 ± 0.53	38.52 ± 1.18	48.52 ± 0.78$^{▲}$
D9-ACBP-KDEL	13.06 ± 0.77	40.54 ± 1.35	46.39 ± 0.72
ACBP	11.48 ± 0.71$^{▼}$	37.20 ± 0.52$^{▼}$	51.31 ± 0.71$^{▲}$
D9-ACBP	12.38 ± 0.16$^{▼}$	39.85 ± 0.51	47.76 ± 0.39

Table A2.3. FA composition of seed oil of *A. thaliana* T_3 seeds transformed with *BnACBP* constructs. Mean mol% ± SD of the means (n=4). ($^{\blacktriangle}$/$^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05; ($^{\Delta}$/◊) values significantly greater/ smaller than WT at α=0.1

Construct	16:0	18:0	18:1	18:2	18:3	20:1
WT	8.19 ± 0.03	3.61 ± 0.04	15.63 ± 0.03	27.52 ± 0.08	17.87 ± 0.10	19.41 ± 0.06
Null Segregate	8.39 ± 0.04	3.27 ± 0.08	15.91 ± 0.71	28.22 ± 0.31	17.41 ± 0.59	19.07 ± 0.30
ACBP-Oleo	9.08 ± 0.36$^{\blacktriangle}$	2.45 ± 0.07$^{\blacktriangledown}$	16.19 ± 1.51	32.11 ± 1.90$^{\blacktriangle}$	16.09 ± 1.62◊	16.55 ± 0.94$^{\blacktriangledown}$
OleoH3P-ACBP	9.22 ± 0.31$^{\blacktriangle}$	2.62 ± 0.02$^{\blacktriangledown}$	16.87 ± 0.99	32.04 ± 0.40$^{\blacktriangle}$	16.02 ± 0.65◊	16.19 ± 0.38$^{\blacktriangledown}$
B82-Oleo-ACBP	8.79 ± 0.39$^{\blacktriangle}$	3.06 ± 0.02$^{\blacktriangledown}$	16.69 ± 0.45	30.20 ± 1.17$^{\blacktriangle}$	15.57 ± 0.62$^{\blacktriangledown}$	17.09 ± 1.35$^{\blacktriangledown}$
ACBP-KDEL	9.60 ± 0.27$^{\blacktriangle}$	2.73 ± 0.13$^{\blacktriangledown}$	17.32 ± 1.12	33.26 ± 1.50$^{\blacktriangle}$	12.45 ± 1.16$^{\blacktriangledown}$	17.81 ± 0.91$^{\blacktriangledown}$
D9-ACBP-KDEL	8.97 ± 0.33$^{\blacktriangle}$	2.65 ± 0.09$^{\blacktriangledown}$	19.09 ± 2.42$^{\blacktriangle}$	31.05 ± 0.29$^{\blacktriangle}$	13.30 ± 1.75$^{\blacktriangledown}$	18.13 ± 0.28◊
ACBP	7.73 ± 0.12◊	2.60 ± 0.21$^{\blacktriangledown}$	17.06 ± 0.65	29.44 ± 0.73$^{\Delta}$	18.85 ± 0.75$^{\Delta}$	16.74 ± 0.71$^{\blacktriangledown}$
D9-ACBP	8.34 ± 0.08	2.51 ± 0.13$^{\blacktriangledown}$	18.88 ± 0.96$^{\blacktriangle}$	30.82 ± 0.71$^{\blacktriangle}$	14.16 ± 1.19$^{\blacktriangledown}$	18.73 ± 0.34

Table A2.4. Composition of FA classes in *A. thaliana* T_3 seeds transformed with *BnACBP* constructs. Mean mol% ± SD of the means (n=4). ($^{\blacktriangle}$/$^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05.

Construct	SFA	MUFA	PUFA
WT	13.91 ± 0.05	39.30 ± 0.10	46.78 ± 0.15
Null Segregate	13.52 ± 0.11	39.38 ± 0.45	47.08 ± 0.36
ACBP-Oleo	13.32 ± 0.42	37.18 ± 1.42	49.48 ± 1.21$^{\blacktriangle}$
OleoH3P-ACBP	13.42 ± 0.32	37.32 ± 1.27	49.25 ± 0.96$^{\blacktriangle}$
B82-Oleo-ACBP	14.11 ± 0.47	38.70 ± 1.04	47.18 ± 0.62
ACBP-KDEL	13.82 ± 0.26	39.35 ± 1.26	46.81 ± 1.00
D9-ACBP-KDEL	13.04 ± 0.50$^{\blacktriangledown}$	41.50 ± 2.18	45.45 ± 1.69
ACBP	11.80 ± 0.31$^{\blacktriangledown}$	38.46 ± 0.97	49.73 ± 0.76$^{\blacktriangle}$
D9-ACBP	12.25 ± 0.21$^{\blacktriangledown}$	41.59 ± 0.80	46.15 ± 0.61

Table A2.5. FA composition of the seed oil from *B. napus* T_1 seeds. Mean mol% ± SD (n=3). (▲/▼) values significantly greater/ smaller than WT at α=0.05; (*) values significantly greater/ smaller than Escape line at α=0.05.

Construct	16:0	18:0	18:1	18:2	18:3	20:0	20:1
Escape	5.11 ± 0.08	1.64 ± 0.05	56.73 ± 0.06	18.88 ± 0.03	12.52 ± 0.13	0.61 ± 0.007	1.31 ± 0.02
ACBP-Oleosin	4.88 ± 0.05	1.42 ± 0.04	54.80 ± 0.96▼	20.17 ± 0.65▲	13.59 ± 0.35▲	0.54 ± 0.02	1.27 ± 0.04

Table A2.6. Composition of FA classes in *B. napus* T_1 seeds. Mean mol% ± SD (n=3). (▲/▼) values significantly greater/ smaller than Escape line at α=0.05.

Construct	SFA	MUFA	PUFA
Escape	7.76 ± 0.12	60.82 ± 0.06	31.40 ± 0.10
ACBP-Oleo	7.20 ± 0.08	59.02 ± 0.90▼	33.76 ± 0.99▲

Table A2.7. FA composition of the seed oil from *A. thaliana* T_2 seeds transformed with *AthACBP_RNAi*(cons) constructs. Mean mol% ± SD of the means (n=10). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05.

Construct	16:0	18:0	18:1	18:2	18:3	20:1
WT	8.31 ± 0.41	3.57 ± 0.36	15.70 ± 0.67	27.49 ± 0.62	17.07 ± 1.01	19.60 ± 0.71
pSBS4148	8.63 ± 0.29	2.42 ± 0.24$^{\blacktriangledown}$	19.25 ± 1.11$^{\blacktriangle}$	30.88 ± 0.85$^{\blacktriangle}$	15.18 ± 0.68$^{\blacktriangledown}$	17.66 ± 0.93$^{\blacktriangledown}$
pSBS4149	8.12 ± 0.26	2.58 ± 0.23$^{\blacktriangledown}$	19.42 ± 0.59$^{\blacktriangle}$	29.48 ± 0.47$^{\blacktriangle}$	14.64 ± 0.44$^{\blacktriangledown}$	19.08 ± 0.61

Table A2.8. Composition of FA classes in *A. thaliana* T_2 seeds transformed with *AthACBP_RNAi*(cons) constructs. Mean mol% ± SD of the means (n=10). ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05.

Construct	SFA	MUFA	PUFA
WT	14.15 ± 0.53	39.80 ± 1.07	46.04 ± 1.36
pSBS4148	12.33 ± 0.52$^{\blacktriangledown}$	40.52 ± 0.77	47.11 ± 0.95
pSBS4149	12.21 ± 0.56$^{\blacktriangledown}$	42.52 ± 0.88$^{\blacktriangle}$	45.26 ± 0.71

Table A2.9. FA composition of the seed oil from *A. thaliana* T_2 seeds transformed with *AthACBP_RNAi*(Nap) construct. mol% ± SD of the means. ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05.

Construct	16:0	18:0	18:1	18:2	18:3	20:0	20:1
WT	8.39 ± 0.08	3.35 ± 0.05	16.41 ± 0.84	27.87 ± 0.35	16.16 ± 0.20	2.22 ± 0.08	19.18 ± 0.25
pGreen1	8.47 ± 0.04	4.41 ± 0.03$^{\blacktriangle}$	15.05 ± 0.01$^{\blacktriangledown}$	24.00 ± 0.09$^{\blacktriangledown}$	18.33 ± 0.02$^{\blacktriangle}$	3.13 ± 0.02$^{\blacktriangle}$	20.09 ± 0.04
pGreen2-12	8.33 ± 0.16	3.14 ± 0.16	17.03 ± 0.76	28.48 ± 0.38	15.93 ± 0.60	2.04 ± 0.09	18.59 ± 0.24

Table A2.10. Composition of FA classes in *A. thaliana* T_2 seeds transformed with *AthACBP_RNAi*(Nap) construct. Mean mol% ± SD of the means. ($^{\blacktriangle}/^{\blacktriangledown}$) values significantly greater/ smaller than WT at α=0.05.

Construct	SFA	MUFA	PUFA
WT	13.96 ± 0.08	40.40 ± 0.41	45.63 ± 0.36
pGreen1	16.02 ± 0.09$^{\blacktriangle}$	40.20 ± 0.06	43.77 ± 0.07$^{\blacktriangledown}$
pGreen2-12	13.52 ± 0.40	40.50 ± 0.81	45.96 ± 0.48