Down-Regulation of *CELLULOSE SYNTHASE* Expression in Arabidopsis Seeds as a Possible Means of Increasing Seed Oil and Protein Content, and Reducing Fibre Content

by

Kethmi Nirmani Jayawardhane Rathnayaka

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in
Plant Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Oleaginous seeds, with higher oil and protein content, have higher economic value in the seed oil industry. Meal resulting from the seed oil extraction process is a widely used protein source in animal feed. The reduction of the complex fibre component of seed tissues may direct more carbon into oil and/or protein biosynthesis, thus resulting in seed with higher oil and/or protein content. Since cellulose makes up a large fraction of fibre within seed meal, reducing the levels of this complex carbohydrate may lead to the improved digestibility of the meal. Partial down-regulation of genes catalyzing cellulose synthesis (i.e., *AtCESA1* and *AtCESA9*) using seed-specific intron-spliced hairpin RNA (RNAi) constructs in Arabidopsis (*Arabidopsis thaliana*) resulted in reductions in the cellulose content of seeds compared to the empty vector control line. Constitutive down-regulation of the *AtCESA9* gene also resulted in reduced cellulose content without impacting the vegetative growth of the plant. These results suggest that down-regulation of cellulose synthesis could be a promising strategy for increasing the digestibility of meal derived from oilseed crops such as canola-type *Brassica napus*. The observed reduction in cellulose content in Arabidopsis, however, did not result in an increase in seed oil content. In contrast, cellulose reduction in *AtCESA1* seed-specific RNAi lines resulted in a slight increase in seed protein content. Based on the results presented, it can be concluded that down-regulation of *AtCESA* genes combined with other metabolic engineering interventions has the potential to result in significantly increased seed oil and/or protein content.

Preface

Drs.Weselake, S. Singer (Department of Agricultural, Food, and Nutritional Science, University of Alberta) and I designed the experiments presented in this thesis. Dr. Singer provided modified RNAi background vectors for the study and I developed the RNAi cassettes and all the plant transformation cellulose synthase RNAi vectors. I performed all experiments and obtained the data for all morphological, seed oil, seed protein and cellulose content studies.

Acknowledgements

I express my gratitude to everyone who supported me throughout my graduate research. First and foremost I would like to express my sincere gratitude to my supervisor Dr.Randall Weselake, for accepting me as his Master's student and giving the opportunity for me to join in such a great group and obtain a rewarding graduate school experience. I'm grateful to him for the continuous support of my research, for his motivation, thoughtful guidance, critical comments and financial support. I thank my committee member Dr. Nat Kav for his constructive criticisms and support during my program. I am very thankful to Dr. Stephen Stelkov to agree to serve as my external examiner.

Countless thanks to Dr. Stacy Singer who was very generous with her time in helping me in numerous ways for giving guidance, sharing experience and motivation. I'm very thankful to Drs. Elzbieta Miekiewska and Saleh Shah for their suggestions and guidance; their enthusiasm for scientific research always inspired me to feel confident, aim high and overcome any difficulties. Many thanks to Drs. Jocelyn Ozga and Rong-Cai Yang for their suggestions in data analysis. I would also like to thank Dr. Kelvin Lien for his technical support in protein analysis for my project. I thank our former lab manager, Annie Wong, for her caring and timely technical support, and assistance with ordering of materials for experimentation. Thanks also to Faculty of Graduate Studies and Research travel awards for supporting my attendance at conferences.

I thank all past and present members of the Weselake group including Chris Kazala, Dr. Geetha Ramakrishnan, Dr. Scott Greer, Dr. Xue Pan, Dr, Champa Wijekoon, Yingyu Lin, Kristian Caldo, Yang Xu, Ziliang Song and Yasmeen El Hajj Abdallah for providing a friendly

and supportive atmosphere at work and also useful feedback and insightful comments throughout my project.

My sincere gratitude goes to my husband Dinuka Waduthanthri for all his support, understanding, encouragement and being there for me always. Most importantly, my heartfelt gratitude goes to my parents for their unreserved love, inspiration, and never ending financial and emotional support throughout my life. Finally, my friend's loyal encouragement and sharing helped keep my life as a graduate student joyous and ever optimistic.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACCase	acetyl-coA carboxylase
AAs	amino acids
ACP	acyl carrier protein
ADF	acid detergent fibre
cDNA	complementary DNA
CDP-choline	cytidine diphosphate-choline
CESA	cellulose synthase
CoA	coenzyme A
COB	COBRA
CPT	CDP-choline:diacylglycerol cholinephosphotransferase
CSC	cellulose synthase complex
CSL	cellulose synthase like
DAG	*sn*-1,2-diacylglycerol
DGAT	diacylglycerol acyltransferase
DIG	digoxigenin
ER	endoplasmic reticulum

FA	fatty acid
FAD	fatty acid desaturase
FAE	fatty acid elongase
FAME	fatty acid methyl ester
FAS	fatty acid synthase
FAT	acyl-ACP thioesterases
FAX1	fatty acid export 1
GPAT	*sn*-glycerol-3-phosphate acyltransferase
G3P	*sn*-glycerol-3-phosphate
HFA	hydroxy fatty acid
INV	invertase
KAS	3-ketoacyl-ACP synthases
KOR	KORRIGAN
LACS	long-chain acyl-CoA synthetase
LPAAT	lysophosphatidic acid acyltransferase
LPC	lysophosphatidylcholine
LPCAT	lysophosphatidylcholine acyltransferase

me^p	malic enzyme
mFA	modified fatty acid
MUFA	Monounsaturated fatty acid
MVBs	multivesicular bodies
NADH	nicotinamide adenine dinucleotide
NUE	nitrogen utilization efficiency
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PBs	Protein bodies
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDAT	phospholipid:diacylglycerol acyltransferase
PDCT	phosphatidylcholine:diacylglycerol cholinephosphotransferase
PDH	pyruvate dehydrogenase
PLA	phospholipase A
PPC	phosphoenolpyruvate carboxylase
PSV	Protein storage vacuoles

PUFA	polyunsaturated fatty acid
SAD	stearoyl-ACP desaturase
SFA	Saturated fatty acid
SSP	Seed storage protein
TAG	triacylglycerol
TED	tracheary element differentiation related
SUS	sucrose synthase
UDP	Uridine diphosphate
UGPase	UDP-glucose pyrophosphorylase
VLCF	very-long- chain fatty acid

1. Introduction

Oilseed crops, such as canola-type *Brassica napus*, are mainly valued for the oil stored in the seeds which serves as a major source of energy and nutrition worldwide (NSW Department of Primary Industries, 2014). Since the demand for the vegetable oil has increased rapidly during past decade with a rapidly increasing human population, numerous attempts have been made to develop molecular strategies to increase seed oil content (Weselake et al., 2009). These genetic engineering approaches have involved studies with the model plant Arabidopsis (*Arabidopsis thaliana*) and oil crops (Singer et al., 2013). In addition to the oil, storage protein in canola seeds also adds considerable value to the crop because it is an important nutritional component of the meal often used as animal feed and in the aquaculture industries (Canola meal feed industy guide, 2009). Conventional breeding has been explored as a means of boosting the seed protein content of the Brassicaceae (Fick, 1983; Slominski et al., 2004). The possible use of genetic engineering to increase seed storage protein content in the Brassicaceae family, however, is rarely mentioned. Further it has been shown that seed oil and protein content are inversely correlated in oilseeds, which apparently is linked to the limited success in obtaining high oil and protein seed in early studies (Fick, 1983). Later, it was found that reducing the fibre content of oilseeds resulted in high oil and high protein oil seed crop varieties (Fick, 1983; Slominski et al., 2004). Reducing the complex fibre content of oilseeds could also provide a secondary benefit in terms of better digestibility, since high-fibre meal limits its usage as an animal feed (NSW Department of Primary Industries, 2014).

.

Cellulose is a major component of plant cell walls which makes up nearly one third of total plant mass (Somerville, 2006). Cellulose is synthesized by a large multimeric complex that contains cellulose synthase subunits (CESAs) as their catalytic domains (Somerville, 2006). In Arabidopsis, these CESA proteins are encoded by 10 cellulose synthase genes (*AtCESA*) and can be divided into two categories depending on whether they are members of complexes that drive primary or secondary wall biosynthesis. *AtCESA1* and *AtCESA9* are two genes that are expressed preferentially in developing seeds (Beeckman et al. 2002). *AtCESA1* is linked to primary cell wall synthesis and mutations in this gene have been found to affect cellulose synthesis, thereby reducing cell wall integrity in rapidly dividing cells (Beeckman et al. 2002; Burn et al. 2002). AtCESA9 was previously reported to be involved in primary cell wall synthesis and partially redundant to other AtCESA isoforms, but later, it was shown to be involved in secondary cell wall synthesis in the seed coat (Stork et al., 2010).

The overall objective of this study was to test the hypothesis that down-regulation of the production of cellulose within seed tissues might re-direct carbon flow to oil and/or protein biosynthesis. In addition, this intervention could potentially increase the quality of the meal due to a decrease in fibre content associated with decreased cellulose content. The above hypothesis was tested in Arabidopsis through the down-regulation of *AtCESA1* or *AtCESA9* using an RNAi interference approach.

The results of the investigation suggested that down-regulation of *AtCESA* genes could be an effective strategy for reducing the insoluble fibre content in the seed. If this strategy is applied to canola in the future, it could potentially result in seed lines which produce a more easily digestible meal. Reducing cellulose content on its own, however, was not effective in increasing seed oil content. In contrast, seed-specific down-regulation of *AtCESA1* resulted in a slight

increase in seed protein content. It is possible that significant increases in seed oil and/or protein content may be achieved in the future by combining down-regulation of *AtCESA* genes with other metabolic engineering interventions.

2. Literature Review

Most annual crops retain their storage reserves within the embryo or endosperm in seeds. During seed development, sucrose derived from photosynthesis is transported from leaves to seeds where it serves as a carbon source for the synthesis of different storage compounds such as starch, oil and protein. The proportion of these storage compounds varies immensely among different plant species. In oilseed crops, starch accumulates in the early stages of embryo development and gradually decreases to undetectable levels by maturity. However, protein and oil contents increase substantially during the later stages of seed development (Andriotis et al., 2010).

Oilseed crops are mainly used for food, feed and industrial applications and the demand for such economic crops has markedly increased in the world during the past few decades. In fact, canola (mainly *Brassica napus*) as a major oleaginous cash crop in Canada generates approximately $19 billion in economic activity and 65%-80% of the seed value comes from seed oil (NSW Department of Primary Industries, 2014; http://www.canolacouncil.org/markets-stats/industry-overview/economic-impact-of-the-canola-industry/). The high fibre, low protein and lower energy content in canola meal compared to soybean meal, however, negatively affects its overall nutrient quality and commercial uses (Canola meal feed industry guide, 2009). Cell wall materials, enriched in cellulose, are generally non-digestible by monogastric animals, thus limiting the effective utilization of the meal (Jiang and Deyholos, 2010).

Cellulose is the major component of plant cell walls, making up nearly one third of the total plant mass (Shi et al., 2008). As a strong carbon sink in plants, the biosynthesis of cellulose recruits a considerable amount of carbon produced by photosynthesis (Somerville, 2006; Weber

et al., 1997). Since biosynthetic processes leading to oil, protein and cellulose share the same carbon source, diverting carbon from cellulose biosynthesis to seed oil/protein production, without a negative impact on plant development has the potential to further increase seed oil and protein content. In addition, reducing the cellulose content of the seed has the potential to increase digestibility of the resulting meal. This literature review discusses carbon partitioning in plants for oil, protein and cellulose production, and examines processes to modify the relevant metabolic pathways to further increase the content of value-added compounds in the seed. The importance of these modifications to the oilseed industry is also addressed.

2.1 Carbon partitioning in oilseed crops

In higher plants, photoassimilate is transported in the form of sucrose and used by the seed to produce storage reserves (Stadler et al., 2005). In seeds, the carbon produced from photosynthesis is partitioned into different storage compounds including starch, protein and oil through complex biosynthetic processes (Figure 2.1). These compounds help to fuel germination and early seedling growth until the developing plant can rely exclusively on photosynthesis as its source of energy (Sanjaya et al., 2011; Shi et al., 2012).

The relative proportions and amounts of different storage reserves in the seed can vary greatly depending on the plant species. Cereals store carbon as starch and protein predominantly in the endosperm while the embryo is enriched in oil. The proportion of oil contributed by the cereal's embryo, however, is rather low because the endosperm represents a large portion of the seed (Ekman et al., 2008). Nonetheless, there are some cereals, such as maize (*Zea mays*) which contain a relatively high amount of oil due to the large size of their embryos (Alexander and Seif, 1963). In contrast, some other cereals such as oats (*Avena sativa*) are capable of producing oil in

the starchy endosperm (Ekman et al., 2008). Oilseeds such as canola and the model oilseed, *Arabidopsis thaliana*, on the other hand, accumulate starch only transiently during early embryo development (Lin et al., 2006).

Given the economic value of seed storage reserves, carbon flux during seed development has been studied extensively (Schwender and Ohlrogge, 2002; Stadler et al., 2005; Baud et al., 2008). Stadler et al. (2005) suggested that in Arabidopsis, sucrose transported through the phloem first unloads to the seed coat and then moves into the embryo via the endosperm. This sucrose is then utilized as the carbon source for starch, acetyl-Coenzyme A (CoA) and amino acid biosynthesis (Figure 2.1) (Shi et al., 2012; Baud et al., 2008).

Analysis of the primary carbon sources available in the liquid endosperm of developing *B. napus* seeds has revealed that the major organic compounds supplied to the developing embryo are sucrose, glucose, Gln, Glu, and Ala (Shewender and Ohlrogge, 2002). Sucrose entering the seed sink can be hydrolyzed by two different pathways catalyzed by two distinct enzymes: invertase (INV) or sucrose synthase (SUS) (Barratt et al., 2009). While INV catalyzes the irreversible hydrolysis of sucrose to fructose and glucose, SUS catalyzes the reversible conversion of sucrose to UDP-glucose and fructose (Barratt et al., 2009). The energy requirement for the INV pathway is relatively high compared to SUS pathway, and the contributions of the two enzymes to sucrose hydrolysis vary between different plant species, tissues and developmental stages (Endler and Persson, 2011). In several species, SUS activity is a marker for storage activity. Furthermore, it has been suggested that among the six members of the *AtSUS* gene family, two isoforms (SUS2 and SUS3) are mainly involved in seed storage compound accumulation (Baud et al., 2008). A subsequent study, however, yielded different findings, wherein these two isoforms were shown to not be essential for storage metabolite

production in *B. napus* developing embryos (Schwender et al., 2003). Similarly, Barratt et al. (2009) disrupted the genes encoding SUS1, SUS2, SUS3 and SUS4 isoforms in Arabidopsis and the resulting plants displayed no deviations in starch or sugar content, seed weight or lipid content, cellulose content, or cell-wall structure. As such, both studies suggest that INV may have the ability to compensate for loss of cytosolic SUS isoforms (Barratt et al., 2009).

The resulting hexoses (glucose and fructose) derived from this enzymatic cleavage generate hexose phosphates that then participate in different pathways, including transient starch biosynthesis in plastids, the oxidative pentose phosphate pathway, and both cytosolic and plastidial glycolytic pathways (Baud et al., 2008). Ultimately, hexose phosphates are converted to pyruvate, which is in turn converted to acetyl-CoA by oxidative decarboxylation catalyzed by the plastidial pyruvate dehydrogenase (PDH) complex (Baud et al., 2008). This plastid-derived acetyl-CoA is used to fuel fatty acid (FA) synthesis. On the other hand, the mitochondrial PDH complex catalyzes the formation of acetyl-CoA that is used in the citric acid cycle. It has been suggested, however, that acetyl-CoA produced in the mitochondria can also be converted into acetate, which may move into plastids where it is converted to acetyl-CoA for use in FA synthesis (Marillia et al., 2003; Weselake et al., 2009).

Cleaved sucrose within the *B. napus* embryo also furnishes 70% of the carbon requirement for storage protein synthesis, while the remaining carbon requirement is derived from directly fluxing amino acids (mainly Gln and Ala) and/or organic acids (malate) (Schwender and Ohlrogge, 2002; Schwender et al., 2006). Indeed, isotopic labeling of storage proteins and lipids in *B. napus* embryonic cultures revealed that in addition to hexoses and sucrose, amino acids are used to deliver both carbon and nitrogen to the growing embryo. Furthermore, it was found that amino acids in the growth medium are incorporated primarily into

storage proteins and do not provide carbon for plastidial FA synthesis (Schwender and Ohlrogge, 2002).

In terms of nitrogen, amino acids supplied from the endosperm to the embryo fulfill most or all of the nitrogen requirements of the developing embryo via their incorporation into proteins. These incoming amino acids act as building blocks for the storage proteins. In addition, they can also act as precursors in different amino acid biosynthesis pathways within the cell (Baud et al., 2008), which occur in both the cytosol and plastids as co-products of the glycolytic pathway. For example, most of the oxaloacetate produced from glycolytic phosphoenolpyruvate (PEP) is used in the synthesis of Asp, and other amino acids are derived from it (Baud et al., 2008).

Carbon derived from sucrose is also used to support cell wall production. Activated glucose in the form of UDP-glucose is used in cellulose production (Endler and Persson, 2011). Even though it has been suggested that SUS is mainly involved in generating UDP-glucose for cellulose production in non-photosynthetic tissues of many plant species, as previously stated, Barratt et al. (2009) proposed that the cytosolic INV pathway was the primary route for carbon production from sucrose in support of cellulose synthesis in non-photosynthetic cells of Arabidopsis (Endler and Persson, 2011; Barratt et al., 2009). Although SUS directly catalyzes the formation of UDP-glucose, the glucose produced by the catalytic action of INV is converted to UDP-glucose by the catalytic action of UDP-glucose pyrophosphorylase (UGPase) (Endler and Persson, 2011).

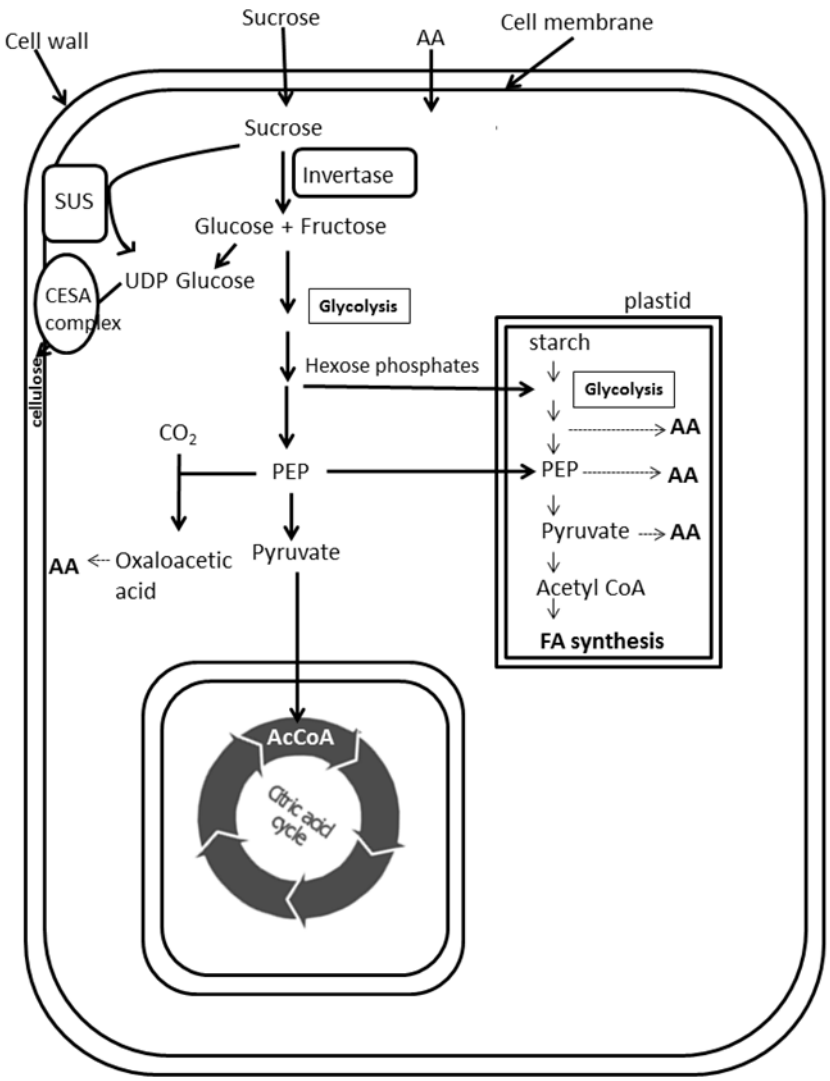

Figure 2.1 Simplified schematic diagram of carbon partitioning in a maturing oilseed embryo (Adapted from Baud et al., 2008)

Cells gain fixed carbon from photosynthesis in the form of sucrose. This sucrose can be hydrolyzed by the catalytic action of two different enzymes. Invertase (INV) action results in the formation of fructose and glucose whereas sucrose synthase (SUS) action results in the formation of UDP-glucose and fructose. The resulting hexoses can be used for starch biosynthesis or processed through the glycolytic pathway in either plastids or the cytoplasm. The pyruvate resulting from plastidial glycolysis subsequently produces acetyl-CoA, which is a precursor for fatty acid (FA) synthesis. In contrast, cytoplasmic pyruvate enters the citric acid cycle resulting in the generation of ATP. Phosphoenolpyruvate (PEP) and pyruvate resulting from plastidial and cytoplasmic glycolysis can also be used to produce amino acids (AAs). UDP-glucose produced through INV or SUS pathways can be used by the cellulose synthase (CESA) complex to catalyze the synthesis of cellulose. SUS directly produces UDP-glucose, whereas INV first hydrolyzes sucrose into glucose and fructose and those glucose are been converted to UDP-glucose.

Having presented information on the utilization of sucrose arriving at the seed sink, the following sections detail the biosynthesis of storage lipids, storage proteins and cellulose, with an emphasis on the Brassicaceae family.

2.2 Storage lipid biosynthesis in plants

Triacylglycerol (TAG) is the predominant storage lipid in the seeds or fruits of most oleaginous plants. In seeds, TAG biosynthesis starts with *de novo* FA synthesis in plastids followed by the export of nascent FAs from the plastid to the cytoplasm. TAG assembly takes place in the ER membrane. Most of the information summarized below is found in Chen et al. (2015), Singer et al. (2013), Chapman and Ohlrogge (2012) and Baud et al. (2008).

2.2.1 Fatty acid biosynthesis

In seeds, FA biosynthesis, which occurs in the stroma of plastids, is the initial phase of storage lipid biosynthesis (Figure 2.2). The initial substrate for FA biosynthesis is acetyl-CoA, which is produced from plastidial pyruvate via the catalytic action of the PDH complex. Acetyl-CoA is converted into malonyl-CoA by the ATP-dependent catalytic action of acetyl-CoA carboxylase (ACCase). Plastidial heteromeric ACCase, one of the two types of ACCases reported so far, is involved in malonyl-CoA formation. This malonyl-CoA furnishes the carbon requirement of each cycle of FA biosynthesis. Before entering into the FA biosynthesis pathway, malonyl-CoA is converted into malonyl-acyl carrier protein (ACP) via the catalytic action of malonyl-CoA: acyl carrier protein malonyltransferase. These malonyl-ACP subunits are then committed to a series of condensation reactions that are catalyzed by fatty acid synthase (FAS), which is an easily dissociable multi-subunit complex consisting of mono-functional enzymes. Two carbon units are added to the growing FA chain sequentially by four reactions, condensation, reduction,

dehydration, and a second reduction. There are three enzymes known as β-ketoacyl-acyl carrier protein (ACP) synthase I, II, and III (KASI, KASII, and KASIII) in plants which are involved in catalyzing the condensation reactions. The initial condensation of acetyl-CoA with malonyl-ACP is catalyzed by β-ketoacyl-ACP synthase III (KASIII) to synthesize 3-ketobutyryl (C4:0)-ACP in the first cycle. KASI catalyzes the condensation of FA chains up to the chain lengths C16 by catalyzing the addition of two carbon units at a time and KASII catalyzes the condensation of those with a chain length of C16 to C18. The resulting β-ketoacyl-ACP is reduced, dehydrated and reduced again by the catalytic action of β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydratase and enoyl-ACP reductase, respectively. Sequential recurrence of these four reactions produces acyl-ACPs with a chain length up to C18. The majority of plastidial FAs consist of C16-ACP and C18:1 (18:1Δ^{9cis})-ACP derived through the desaturation of C18:0-ACP catalyzed by a stromal stearoyl (C18:0)-ACP desaturase. Subsequent hydrolysis of these acyl-ACPs is catalyzed by acyl-ACP thioesterase (FAT), which releases free FAs that can then move across the inner plastidial membrane with the assistance of fatty acid export (FAX)1 (Li et al., 2015). Free FAs are subsequently converted into acyl-(CoA) esters on the outer membrane of the plastid through the catalytic action of acyl-CoA synthetase. The nascent C16:0, C18:0 and C18:1 acyl-CoAs can then serve as acyl donors in the "eukaryotic pathway of TAG synthesis" in the endoplasmic reticulum (ER) membrane (Bates and Browse, 2011).

Cytoplasmic acyl-CoAs can be further elongated via the catalytic action of fatty acid elongase (FAE), which is an ER membrane-bound multi-enzyme complex driving the extension of the FA chain via a series of enzymatic reactions in a similar way to *de novo* FA synthesis. This process produces very long chain fatty acids (VLCFAs). Linoleic (C18:2$\Delta^{9cis,12cis}$; hereafter C18:2) and α-linolenic (C18:3^{9cis,12cis,15cis}; hereafter C18:3) acids account for 45-50% of the total

FA content of Arabidopsis seed oil. The desaturation reactions that produce 18:2 and 18:3 from 18:1 are catalyzed by fatty acid desaturase (FAD)2 and FAD3, respectively, which act on acyl chains on phosphatidylcholine (PC; Figure 2.4; Okuley et al., 1994). Hydroxylation and other modifications of 18:1 also occur at the level of PC in oleaginous plants (Chen et al., 2015).

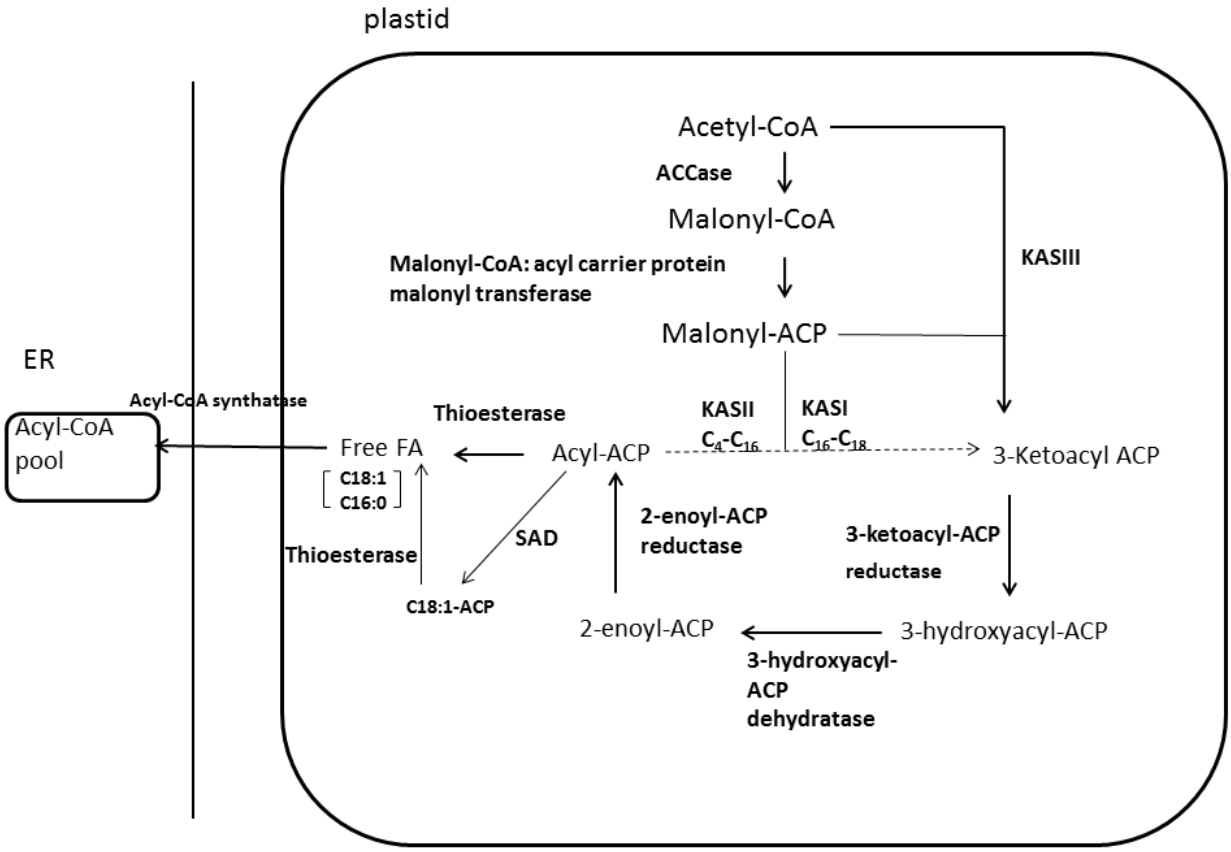

Figure 2.2 *de novo* fatty acid synthesis (adapted from Singer et al., 2013)

FA synthesis is initiated in the plastid with the conversion of acetyl-CoA into malonyl-CoA by the catalytic activity of acetyl-CoA carboxylase (ACCase). Subsequently, malonyl-CoA:ACP malonyl trasferase catalyzes the conversion of malonyl-CoA into malonyl-acyl carrier protein (ACP). Acyl-ACP is subjected to elongation in successive cycles and this requires catalytic activities of ketoacyl-ACP synthases (KAS), ketoacyl-ACP reductase, hydroxyacyl-ACP dehydrase and enoyl-ACP reductase for condensation, reduction, dehydration and reduction respectively. In the initial cycle, malonyl-ACP subjected to condensation with acetyl-CoA by the catalytic action of (KAS) III which produces the 3-ketobutyryl (4:0)-ACP. KAS I and KAS II catalyze the condensation in cycles from 4:0-ACP to 16:0-ACP and 16:0-ACP to 18:0-ACP, respectively, using malonyl-CoAs as acyl-donors. Each condensation reaction produces 3-Ketoacyl-ACP, which is subsequently subjected to reduction, dehydration and reduction again to produce 3-hydroxyacyl-ACP, 2-enoyl-ACP and Acyl-ACP respectively. Stearoyl (18:0)-ACP desaturase (SAD) catalyzes the conversion of 18:0-ACP to 18:1-ACP. Thioesterases then catalyze the cleavage of the acyl chains from ACP to produce free fatty acids, which are exported to the cytosol and immediately converted into acyl-CoAs on the outside of the plastid.

2.2.2 Triacylglycerol assembly

TAG biosynthesis occurs on the ER membrane. The Kennedy pathway (Figure 2.3) utilizes *sn*-glycerol-3-phosphate (G3P) as a glycerol backbone for acyl-CoA-dependent acylation (Bates and Browse, 2011). This pathway involves the sequential acylation of the *sn-1*, *sn-2* and *sn-3* positions of the G3P backbone with three fatty acyl moieties. The first acylation, which incorporates a fatty acyl moiety to the *sn-1* position of G3P to produce lysophosphatidic acid (LPA), is catalyzed by *sn*-glycerol-3-phospate acyltransferase (GPAT). In turn, lysophosphatidic acyltransferase (LPAAT) utilizes LPA as its substrate and catalyzes the second acylation at the *sn-2* position to form phosphatidic acid (PA). Subsequently a phosphate group is removed from PA through the catalytic action of phosphatidic acid phosphatase (PAP) to produce *sn* -1, 2-diacylglycerol (DAG) followed by a final acylation at the *sn*-3 position to produce TAG. This final acylation is catalyzed by diacylglycerol acyltransferase (DGAT) (Weselake et al., 2009). The Kennedy pathway is a major pathway for oleaginous plants which produce TAG enriched in saturated and monounsaturated FAs such as palm and coconut (Chen et al., 2015).

In plants that produce substantial levels of polyunsaturated fatty acids (PUFAs) in their seed oils, DAG synthesized in the Kennedy pathway can also be converted to PC by the catalytic action of CDP-choline: diacylglycerol cholinephosphotransferase and phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT; Figure 2.4; Chen et al., 2015; Singer et al., 2013; Chapman and Ohlrogge, 2012; Yang et al., 2012). As mentioned previously, PUFAs are formed on PC by the catalytic action of FAD2 and FAD3. The PUFA-enriched acyl chains enter the acyl-CoA pool through various mechanisms, including acyl-exchange, or the PUFA-enriched DAG skeleton of PC can enter the Kennedy pathway through phosphocholine head group exchange catalyzed by PDCT (Chapman and Ohlrogge, 2012; Singer

et al., 2013).

In addition, TAG can be synthesized via an acyl-CoA-independent pathway which involves the transfer of an acyl group from PC to DAG by the catalytic action of phospholipid: diacylglycerol acyltransferase (PDAT; Figure 2.4). In this case, an acyl group from the *sn-2* position of PC is transferred to the *sn*-3 position of DAG to form TAG (Weselake et al., 2009).

The ratio of TAG formed through *de novo* DAG produced in the Kennedy pathway to PC-derived TAG varies between plant species (Bates and Browse, 2011). The TAG synthesized through these complex pathways accumulates in subcellular structures referred to as oil bodies which pinch off of the ER. The oil bodies are encapsulated by a phospholipid monolayer and embedded proteins including oleosins (Siloto et al., 2006).

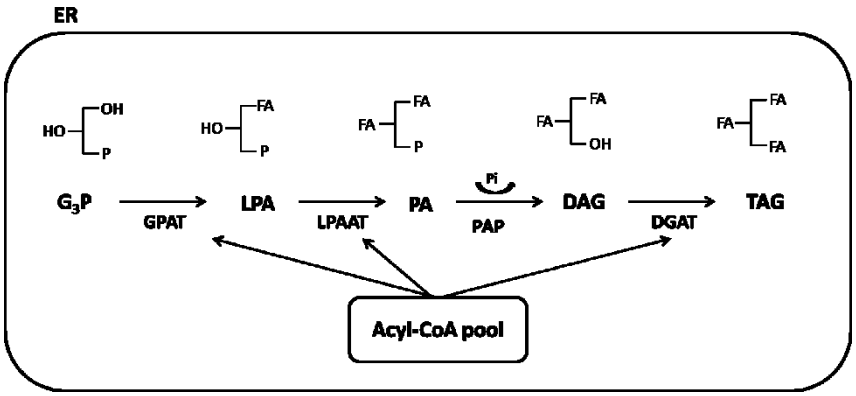

Figure 2.3 Kennedy pathway for triacylglycerol (TAG) assembly on the endoplasmic reticulum (ER) membrane

The major pathway which has been proposed for TAG biosynthesis is known as the Kennedy pathway. This pathway involves sequential acylation of glycerol-3-phosphate with three fatty acid moieties from acyl-CoA. Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first acylation of the *sn-1* position of *sn*-glycerol-3-phosphate (G3P) to produce lysophosphatidic acid (LPA), followed by the 2nd acylation of the *sn-2* position of LPA to produce phosphatidic acid (PA), which is catalyzed by lysophosphatidic acid acyltransferase (LPAAT). Subsequently, PA is converted into *sn*-1, 2-diacylglycerol (DAG) by the catalytic action of phosphatidic acid phosphatase (PAP). In the final step, diacylglycerol acyltransferase (DGAT) catalyzes the acylation of the *sn*-3 position of DAG to produce TAG.

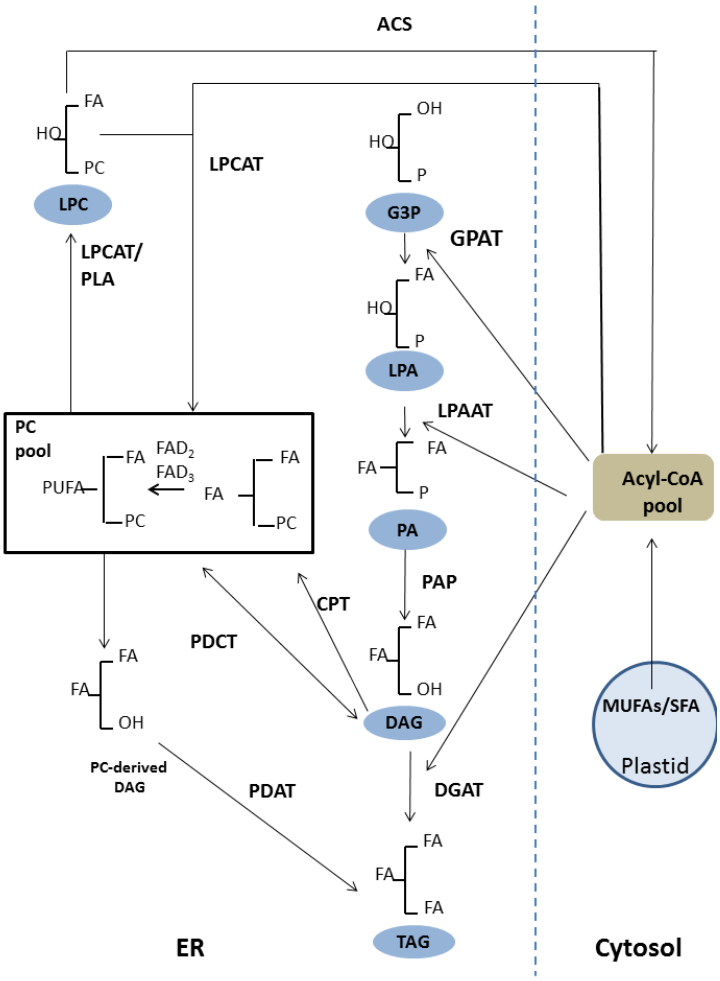

Figure 2.4 Movement of polyunsaturated (PUFA) into the acyl-CoA and DAG pools via the phosphatidylcholine (PC) pool and incorporation into triacylglycerol (TAG)

The *sn*-1, 2-diacylglycerol (DAG) produced via the Kennedy pathway can also be converted to phosphatidylcholine (PC) through the action of *sn*-1,2-diacylglycerol:cholinephosphotransferase (CPT) and/or phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT). Acyl chains present in phosphatidylcholine (PC) can then be desaturated through the catalytic action of fatty acid desaturase (FAD) 2 and FAD3, which results in the production of poly unsaturated FA(PUFA). DAG bearing PUFAs derived from PC returned into linear part of the G3P pathway by the action of PDCT and can be converted to triacylglycerol (TAG). Phospholipid:diacylglycerol acyltransferase (PDAT) catalyzes the transfer of FA at the *sn*-2 position of PC for DAG to generate TAG. PC bearing a PUFA can also release a PUFA from the *sn*-2 position via the catalytic action of phospholipase A 2 (PLA 2) and convert it into acyl-CoA through the involvement of acyl-CoA synthetase (ACS). In addition, acyl-CoA:lyso-phosphatidylcholine acyltransferase (LPCAT) may catalyze acyl-exchange between the *sn*-2 position of PUFA-enriched PC and the acyl-CoA pool. Other abbreviations: G3P, glycerol 3-phosphate;GPAT, Glycerol-3- phosphate acyltransferase LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; MUFA, mono unsaturated fatty acid; SFA, saturated fatty acid

2.3 Seed storage protein biosynthesis

Seed storage protein (SSP) synthesis depends on sucrose and amino acids unloaded into sink tissues from source tissues (Fujiwara et al., 2002). SSP can be distinguished from other plant proteins (housekeeping proteins) by a number of characteristic features including their amino acid composition, tissue specificity, arrangement within the cell and molecular weight (Higgins, 1984). Usually, storage proteins are rich in Asn, Gln, and Arg or Pro and most of them are higher molecular weight, hydrophobic oligomers. The presence of small molecular weight water soluble SSPs has also been reported in various plant species including castor bean (*Ricinus communis*), peanut (*Arachis hypogaea*), pea (*Pisum sativum*) and mung bean (*Vigna radiate*) (Higgins, 1984). Furthermore, all SSP fractions consist of a mixture of polymorphic proteins both within single genotypes and among genotypes of the same species. These polymorphisms mainly arise due to the presence of multigene families and post-translational modifications (Shewry et al., 1995).

In Brassicaceae species, including oilseed rape and Arabidopsis, the major SSPs are napin (2S albumin) and cruciferin (12S globulin) (Herman & Larkins, 1999). The ratio of napin to cruciferin, however, varies immensely among different species within the Brassicaceae family. In *B. napus,* napin is encoded by 10-16 genes while 9-12 genes encode cruciferin (Wanasundara, 2011).

SSP synthesis begins around the fifth week after anthesis with maximum rates of synthesis occurring between weeks 5 and 7 in Brassicaceae species (Murphy et al., 1989). SSPs are synthesized on the rough ER membrane as precursor forms using amino acids and then transferred to protein storage vesicles (PSV) (Higgins, 1984). SSPs first enter the ER lumen after

cleavage of an N-terminal signal peptide (Herman & Larkins, 1999). Further processing of these polypeptides, which includes folding and oligomerization, is facilitated by luminal chaperones and various enzymes leading to the formation of complex proteins as dimers, trimers, and tetramers in the ER lumen. At least three different SSP-trafficking pathways from the ER to PSVs have been observed: 1) the Golgi-dependent dense vesicle mediated pathway, 2) the direct ER-to-PSV transport pathway and, 3) the autophagic pathway (Otegui et al., 2006). It has been suggested that the prominent SSP-trafficking pathway in Brassicaceae is the Golgi-dependent vesicle mediated pathway (Otegui et al., 2006). For example, in Arabidopsis, SSPs synthesized within the ER lumen have been found to be trans-located to the specialized marginal buds of Golgi cisternae and they form dense aggregates. As these buds are transported through stacks of the Golgi from the cis face to the trans face, the buds containing protein aggregates give rise to electron-dense vesicles (DVs). These DVs fuse with vesicles containing protein processing enzymes to form multivesicular bodies (MVBs), which deliver their contents to PSVs (Baud et al., 2008). SSP-trafficking routes vary between plant species, tissues, and developmental stages as well as for different SSPs. In fact, prolamin storage proteins from cereals first aggregate within the ER and are then transported to PSVs via protein bodies (PB), which are ER-derived vesicles (Herman and Larkins, 1999).

A number of studies suggest that SSP biosynthesis and trafficking are very complex processes that are controlled by different receptors (RMR1 and VSR-1) present in the ER, Golgi and vacuoles (Baud et al., 2008). These receptors are involved in protein sorting and determining where and how to translocate precursor forms of the proteins (Herman and Larkins, 1999). Mature seeds contain densely packed and complex protein-filled PSVs that are used to fulfill the nutrient requirements of growing seedlings.

2.4 Cellulose biosynthesis in plants

The biosynthesis of cell wall components, including cellulose, also requires high amounts of carbon produced from photosynthesis. Cellulose is the most abundant cell wall component of plant cell walls, although its amount varies widely in different plant species (Haigler et al., 2001). For example, the cellulose content of dry matter from forage grasses is only 28-30%, while it is 42-45% in woody plants.

2.4.1 Significance of cellulose in plant development

The significance of cellulose in plant development explains its abundance in the plant cell wall as its foundational polymer. The cell wall not only provides protection against environmental stress, but it also provides strength and rigidity to the cell as well as the entire plant body (Keegstra, 2010). Furthermore, cell walls constrain the direction of plant morphogenesis and confer specialized functions such as water conduction, support and control of transpiration (Haigler et al., 2001)**.**

The plant cell wall consists of a primary wall and secondary wall (Keegstra, 2010). The primary walls are formed in growing cells and mainly consist of cellulose, hemicelluloses, pectin and some structural proteins. Secondary walls are the cell walls that are formed after cell growth (enlargement) has ceased and are located between primary cell wall and plasma membrane of the cell. They mainly consist of cellulose and other cell wall materials including lignin, polysaccharides, glycoproteins and waxes which can vary in proportion among different cell types (Keegstra, 2010). Generally, both of these walls mainly consist of cellulose microfibrils, which are made of interconnecting parallel unbranched β-1,4-glucan chains stabilized by intra-chain and inter-chain H bonding, and Van der waals forces (Endler and Persson, 2011). The

primary wall consists of about 25% cellulose on a dry weight basis while in some species, such as cereals, this can be largely variable (Keegstra, 2010; Endler and Persson, 2011). These cellulose microfibrils are embedded in a highly hydrated matrix while hemicellulose connects these microfibrils to each other and forms a load-bearing network around the cell. Because cells tend to expand their size along the direction of least resistance, cellulose deposition in the cell assists in the regulation of the direction of cell wall expansion and growth of the entire organ (Burn et al., 2002). Since cellulose microfibrils are relatively stiff structures, due to extensive non-covalent bonding present between adjacent glucans, they are the main contributors to the strength of the cell wall. On the other hand, cellulose microfibrils are very stable and relatively resistant to enzyme degradation due to a tight structural arrangement that effectively excludes water (Keegstra, 2010).

2.4.2 Cellulose biosynthesis mechanism in plants

2.4.2.1 Cellulose synthase complex (CSC)

Cellulose is synthesized by large multimeric complexes in the plasma membrane referred to as the cellulose synthase complex (CSC), which contains cellulose synthase subunits (CESAs) as catalytic domains (Figure 2.5). These CESA proteins belong to the family 2 glucosyltransferases, which are involved in catalyzing the synthesis of structures composed of repeating β-glycosyl units. Each CESA polypeptide is composed of 8 transmembrane domains, a catalytically active domain and a pair of N-terminal zinc finger domains (Richmond and Somerville, 2000). The transmembrane domains collectively create a pore in the plasma membrane and this pore is known to secrete a single glucan chain into the cell wall (Endler and Persson, 2011). CSCs have been identified as six-lobed rosettes 20-30 nm in diameter as observed in freeze-fracture labeling electron microscopy studies (Brown JR, 1996; Kimura et al., 1999). Each of the lobes is

proposed to be comprised of six CESA proteins, which function as catalytic units for cellulose production, with each CSC encompassing 36 (6×6) individual units that can produce 36 glucan chains simultaneously (Figure 2.5) (Endler and Persson, 2011). It has been suggested, however, that even though the model implies it can produce 36 glucan chains, it is not always true with regards to the number of glucan chains that are observed in each microfibril, which is 18 or less (Kennedy et al., 2007). Several lines of evidence have shown that at least three types of related proteins (isoforms) are necessary to form a functional CSC (Persson et al., 2007; Desprez et al., 2007; Taylor et al., 2003). Each lobe of the CSC consists of three different CESA proteins which are dimerized by two zinc fingers of the CESA proteins and organized into rosette subunits bearing six proteins (Cosgrove, 2005).

CESA proteins are encoded by 10 cellulose synthase genes (*AtCESA*) in Arabidopsis, and can be divided into two categories depending on whether they are members of complexes that drive primary or secondary wall biosynthesis (Somerville, 2006). Of these 10 AtCESA proteins in Arabidopsis, AtCESA1, AtCESA2, AtCESA3, AtCESA5, AtCESA6 and AtCESA9 have been shown to be associated with the primary cell wall CESA complex, while the secondary cell wall CESA complex comprises AtCESA4, 7 and 8 (Persson et al., 2007; Somerville, 2006; Taylor et al., 2003). The biological role of AtCESA10 remains unclear (Kumar and Turner, 2015). Indeed, using co-immunoprecipitation assays, Persson et al. (2007) and Desprez et al. (2007) found that AtCESA1 and 3 are the essential components of the primary CSC, together with some combination of AtCESA2, AtCESA5, AtCESA6 or AtCESA9. Furthermore, they determined that removal of AtCESA1 and 3 components resulted in gametophytic lethality, while null mutations of AtCESA6 can be compensated by AtCESA2 and 5, which are partially redundant to AtCESA6 (Persson et al., 2007; Desprez et al., 2007). AtCESA9 has also been shown to share a high level

of sequence identity with AtCESA6, suggesting that this isoform is redundant to the AtCESA6-related AtCESAs (Endler and Persson, 2011). Furthermore, Beeckman et al. (2002) observed that loss of AtCESA2 and AtCESA9 function yielded relatively minor consequences in primary wall synthesis in Arabidopsis embryogenesis, which indicates that loss of function of AtCESA9 can be compensated by other related isoforms. However, in a later study, it was argued that AtCESA9 may also be involved in secondary cell wall cellulose deposition in the Arabidopsis seed coat (Stork et al., 2010; Somerville, 2006). In addition, Persson et al. (2005) suggested that primary and secondary *AtCESA*s do not express coordinately since primary walls and secondary walls are formed at two different developmental stages within the cell. Primary *AtCESA*s are believed to be expressed from the beginning of cell formation to soon after the end of cell expansion, while secondary CESAs are expressed from later stages of cell expansion to the cell death (Persson et al., 2005). Subsequently, it was suggested that primary and secondary AtCESAs can interact with each other and there is a possibility of forming mixed complexes of primary and secondary AtCESAs at particular times (Carroll et al., 2012).

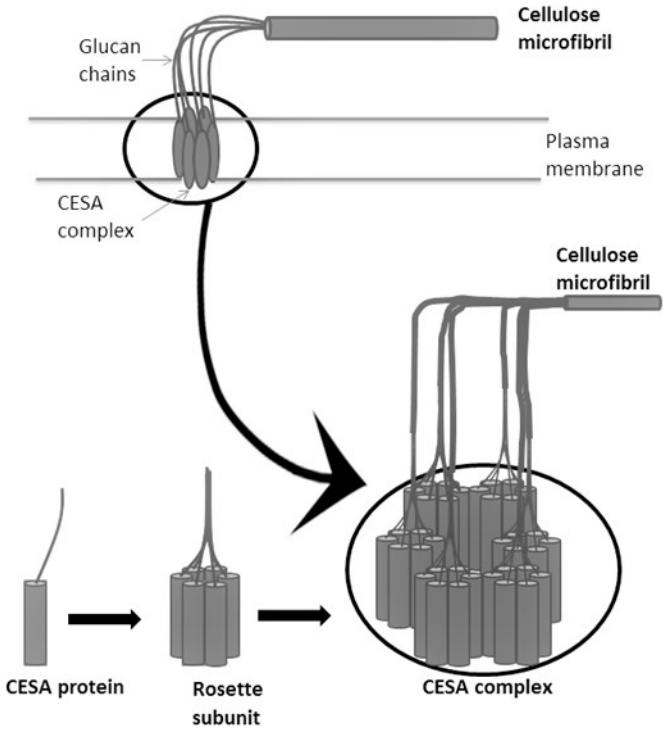

Figure 2.5 Simplified schematic diagram of cellulose synthesisizing machinery

CESA complexes are localized in the plasma mebrane and comprise 6 subunits. In the proposed model there are six CESA protein units including 3 different CESAs for each subunit. Each CESA protein in this rossette structure can produce a single (1,4)-linked β-D-glucan chain; cellulose is formed as a crystalline microfibril that is composed of many such glucans. The resulting cellulose microfibrils are then released for cell wall prodction (adapted from Cosgrove, 2005).

This suggests that the role of different CSC-associated proteins and their interactions are very complex and in need of further characterization.

Apart from CESAs, there are several other proteins involved in cellulose synthesis including cellulose synthase like (CSL), tracheary element differentiation related (TED), COBRA (COB) and KORRIGAN (KOR) proteins. CSL and TED proteins directly interact with at least one CESA protein in either primary or secondary CSCs (Endler and Persson, 2011). The precise function of KOR and COB have not yet been elucidated although it has been suggested that the function of these two proteins in cellulose deposition could be indirect since none have been found to be directly associated with CSCs (Endler and Persson, 2011).

2.4.2.2 Cellulose synthase genes involved in Arabidopsis embryogenesis

The 10 *At*CESA proteins in Arabidopsis are encoded by a family of genes consisting of 10 *AtCESA* genes, namely *AtCESA1-10*. Over the years, emerging data suggests that at least some *CESA* genes may have spatially or temporally-specific expression patterns. Phylogenetic analysis has shown that the clustering of these proteins from different plant species share a cell-specific predictive value (Haigler et al., 2001; Stork et al., 2010). However, some studies claim that at least some of these genes are expressed either temporally or spatially and some studies also suggest that the AtCESA proteins may be functionally redundant in some tissues (Stork et al., 2010; Beeckman et al., 2002). Stork et al. (2010) proposed that *AtCESA9* is seed coat-specific or at least that the AtCESA9 protein is preferentially active in seed coat tissues since T-DNA mutations in *AtCESA9* only affected seed coat cellulose synthesis.

A reverse genetic study in Arabidopsis provided evidence that only four of the 10 *AtCESA* genes, *AtCESA1*, *AtCESA2*, *AtCESA3* and *AtCESA9*, are significantly expressed in the

developing Arabidopsis embryo (Beeckman et al., 2002). Expression studies of the 10 *AtCESA* genes in embryos, young plants, stems and flowers provided evidence that all *AtCESA* genes were expressed in all of these tissues. However, *AtCESA1*, *AtCESA2*, *AtCESA3* and *AtCESA9* were expressed at significantly higher levels in the embryo. Furthermore, *AtCESA5* was found to be expressed at only very low levels using reverse transcription (RT)-PCR analysis, although it was below the detection threshold using digoxigenin (DIG)-labeled RNA through non-radioactive *in situ* hybridization assay of Arabidopsis embryonic tissues (Beeckman et al., 2002). These data suggested that *AtCESA1*, *AtCESA2*, *AtCESA3* and *AtCESA9* are the genes which are involved in primary wall synthesis in Arabidopsis seeds and that down-regulation of these genes may affect cellulose synthesis in seeds.

Previous studies have shown that *AtCESA1* and *AtCESA3* are non-redundant essential genes for cellulose synthesis, since knock-out mutations of these genes caused severe morphological changes in different plant organs (Persson et al., 2007; Beeckman et al., 2002). Mutations in *AtCESA1* and *AtCESA3* can affect cell expansion and cell division. In fact, the antisense lines of these two genes resulted in plants with smaller leaves, shorter stems and smaller floral organs, although the severity of the phenotypes was much weaker than null-mutation *AtCESA1* plants (*rsw1*) (Burn et al., 2002).

Beeckman et al. (2002) have argued that loss of *AtCESA1* gene function does not severely affect early embryogenesis in Arabidopsis. Furthermore, they observed that a *AtCESA1* null mutation (*rsw1*-20) restricts cell expansion rather than affecting cell division in the Arabidopsis embryo, thereby affecting the shape of the developing embryo changing it from anisotropic to isotropic (Beeckman et al., 2002). This occurs due to unregulated cell expansion of the embryo as described by Gillmor et al., (2002). Typically, Arabidopsis embryos are approximately four

times as long at the heart stage than the torpedo stage since its growth primarily occurs in the longitudinal direction. This differential reinforcement of embryonic cells is regulated by the cellulosic cell walls in the epidermis. When this regulated expansion does not occur in the embryonic cells, the overall shape is maintained as a sphere in coordination with increasing volume (Gillmor et al., 2002). *AtCESA2*, *AtCESA3* and *AtCESA9* are co-expressed with *AtCESA1* in the Arabidopsis embryo while *AtCESA1* down-regulation cannot be compensated for by any of these genes even when overexpressed with strong promoters (Burn et al., 2002). *AtCESA2* and *AtCESA9* share high sequence similarity while *AtCESA9* is known to be involved in cellulose biosynthesis in seed coat cells (Stork et al., 2010). A *AtCESA9* T-DNA mutation caused a 25% reduction in the cellulose content of seeds without affecting the cellulose content in other tissues. However, the reduced cellulose content did not show any observable morphological defects in the embryos (Stork et al., 2010). Furthermore, Stork et al. (2010) observed that the *AtCESA9* mutant exhibited depletion of secondary wall biosynthesis in the radial cell walls of epidermal seed coats. These results suggested that even though *AtCESA9* is expressed in Arabidopsis embryos, it may either be functionally active in the seed coat or *AtCESA2* may compensate for the *AtCESA9* defects in the Arabidopsis embryo.

2.4.2.3 Synthesis of cellulose microfibrils

UDP-glucose is the activated sugar donor for β-1,4 glucan chain polymerization. As indicated previously, UDP-glucose is synthesized by either INV-dependent or SUS-dependent pathways (Endler and Persson, 2011).

In two different studies, sistosterol-β-glucoside has been proposed as the primer for cellulose biosynthesis or it positively influences the membrane environment to initiate cellulose

polymerization (Peng et al., 2002; Schrick et al., 2004). CESA enzymes in the CSC use sterols, which are common membrane lipids in plant cells as primers for cellulose microfibril biosynthesis. Thereafter, the CSC continues to catalyze the addition of UDP-glucose residues to the growing glucan chain (Cosgrove, 2005). Each microfibril consists of dozens of crystallized β-1,4- glucan chains, which are 3–5 nm wide and many micrometers in length. This enables the polymer to wind around the cell many times (Cosgrove, 2005). It has been suggested, however, that synthesized β-1,4- glucan chains cannot be directly crystallized into cellulose microfibrils, but prior to crystallization, sterol residues need to be removed from the nascent glucan chain. According to Peng et al. (2002), the KOR enzyme is involved in this removal process. Nevertheless, it is not clear whether there is a direct association of KOR with CSC because many proteins are involved in cellulose synthesis during primary and secondary wall formation (Peng et al., 2002).

2.5 Diverting carbon from other sources to oil and protein biosynthesis, and relevance to the oilseed industry

Seed oil, which is mainly composed of TAG, is one of the major storage compounds that accumulates during seed development in higher oleaginous plants (Shi et al., 2012). These oils are in great demand since they are widely used for food and feed. There is also a growing trend to use vegetable oils as a sustainable replacement for petrochemicals in the form of biodiesel, various polymers and lubricants (Dyer et al., 2008; Sanjaya et al., 2011). Indeed, in Canada alone, a 50%-75% increase in vegetable oil production will be required over the next few years to fulfill the growing demand (Weselake et al., 2009). The oilseed industry accounts for approximately $19 billion economic activity in Canada. Increasing canola oil content by one percentage point would add approximately $90 million of additional value per year to the oilseed

extraction and processing industry in Canada (http://www.canolacouncil.org/markets-stats/industry-overview/). A rapidly increasing human population coupled with limitations on arable land suggests that it will be challenging to produce enough oil for future populations. Therefore, generating oil crops with high seed oil content and quality has become of paramount importance in recent years (Kim et al., 2014; Shockey et al., 2015; Singer et al., 2016).

Along with oil, the protein content of these seeds also plays a crucial role in terms of the animal feed and aquaculture industries (NSW Department of Primary Industries, 2014). Oil-free canola meal usually contains 35%- 50% protein with an excellent balance of amino acids but with some anti-nutritional factors including a relatively high amount of fibre (Tan et al., 2011). Similar to most vegetable protein sources, canola meal is limiting in Lys but contains high levels of Met and Cys. Furthermore, there is a growing tendency regarding the use of these proteins for human consumption due to their well-balanced amino acid composition even though the incorporation of canola meal into food for human consumption, is also limited by the presence of anti-nutritional factors such as sinapine, glucosinolates and tannins (Yoshie-Stark et al., 2008).

Increasing the flow of carbon into oil or protein represents a useful strategy to increase seed value (Ekman et al., 2008; Iyer et al., 2008; Shi et al., 2012; Tomlinson et al., 2004). Ekman et al. (2008) investigated carbon partitioning between oil and carbohydrate in developing oat (*Avena sativa* L.) seeds and found that there was a high oil accumulation in some oat varieties due to a higher proportion of carbon partitioning into oil during seed filling, predominantly at the earlier stages of kernel development. With these results and using global gene expression studies, the investigators further suggested that there is a possibility of redirecting the fixed carbon into oil synthesis by engineering the lipid biosynthetic pathway (Ekman et al., 2008). In another study, comparison of the effect of temperature on protein and oil biosynthesis in developing

soybean (*Glycine max*) cotyledons suggested that cytosolic phosphoenolpyruvate carboxylase (PPC) and malic enzyme (me^p) had fundamental impacts on carbon partitioning into protein and oil in soybean cotyledons. Furthermore, they have suggested that these two enzymes catalyze the anaplerotic reactions of carbon flux to protein and oil in soybean (Iyer et al., 2008). Thus, the investigators proposed that by manipulating these genes it could be possible to increase carbon flow into the plastidic pyruvate pool, enabling increased lipid and protein production. Lin et al. (2012) have succeeded in producing more oil in Arabidopsis seeds by down-regulating the expression of a transcription factor (GLABRA2) that is involved in regulating mucilage production. The investigators down-regulated the *GLABRA2* gene in Arabidopsis, which in turn resulted in down-regulation of the expression of *MUCILAGE MODIFIED 4* (*MUM4*) encoding rhamnose synthase required in mucilage synthesis. This modification caused higher oil production in Arabidopsis seeds due to diverted carbon flux from mucilage biosynthesis to oil biosynthesis (Shi et al., 2012). Similar results were obtained in Arabidopsis vegetative tissues by down-regulating a starch biosynthesis gene (*APS1*) in combination with over-expression of *WRINKLED1,* a gene encoding a transcription factor that up-regulates a number of enzymes involved in FA biosynthesis (Sanjaya et al., 2011). Increased protein content in wheat (*Triticum aestivum*) grains by increasing sucrose uptake to the endosperm has been reported by Weichert et al. (2010). In this study, they overexpressed the sucrose transporter *HvSUT1* (*SUT*) gene from barley (*Hordeum vulgare*) under the control of the endosperm-specific Hordein B1 promoter, and observed increased levels of grain protein accumulation (Weichert et al., 2010). In another study, it has been reported that overexpression of an amino acid permease, *VfAAP1*, increases amino acid and total seed protein content in pea embryos (Weigelt et al., 2008). This intervention, however, that led to deregulation of the carbon-nitrogen balance and the investigators suggested

that there is a general carbon limitation for amino acid/seed protein synthesis (Weigelt et al., 2008). Increasing storage protein content in oil seeds of Brassicaceae by diverting carbon flow from fibre biosynthesis has been achieved through several breeding approaches (Slominski et al., 2004; Fick, 1983). Collectively, these results suggest that oil and/or protein content of oilseed crops can be boosted by altering carbon partitioning.

Excellent amino acid composition, mineral composition and reduced level of glucosinolate content in canola meal make it attractive for food formulations (Canola meal feed industry guide, 2009). A relatively high level of indigestible fibre content, however, limits the feed value of canola meal compared to soybean meal. A higher level of acid detergent fibre (ADF) in canola meal lowers the available energy content of the meal (Canola meal feed industry guide, 2009). ADF is the least-digestible fibre portion of the meal because it mainly consists of cellulose, lignin, silica and insoluble forms of nitrogen (Canola meal feed industry guide, 2009). Therefore, redirecting carbon flow from fibre to seed oil/ protein biosynthesis would be a logical way of producing high quality seed oil crops with high oil/protein and reduced fibre.

3. Materials and Methods

3.1 Plant growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 seeds were cold-treated at 4ºC in the dark for 3 days prior to sowing on sunshine #4 potting mix (Sun Gro Horticulture, Vancouver, Canada). Plants were grown from sowing onwards in a growth chamber at 22ºC with a photoperiod of 18 h day/6 h night and 250 µmol/m2•s light intensity. T_1 and T_2 transgenic plants were grown in 36 inserts and one week after transferring to soil from the selection plates, plants were supplemented with N:P:K 20:20:20 fertilizer once a week (Chissa-Asahi Fertilizer Co., Ltd, Tokyo, Japan)

3.2 Cloning of *AtCESA1* and *AtCESA9* cDNA fragments (320 bp and 371bp respectively) from Arabidopsis

3.2.1 Sequence identification of *AtCESA1* and *AtCESA9*

Cellulose synthase genes that are expressed in Arabidopsis seed embryos were identified using "The Arabidopsis information resource" (TAIR) (https://www.arabidopsis.org). *AtCESA1* (1009057425) and *AtCESA9* (1009039433) cDNAs were selected as target sequences. A 320-bp nucleotide sequence specific to *CESA1c* DNA and a 371-bp nucleotide sequence specific to *CESA9* cDNA were selected using the Geneious Blast search operation (Geneious Basic 5.6.5).

3.2.2 Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from developing siliques of Arabidopsis wild-type (Col-0) plants that had been grown in a greenhouse at 22ºC - 25ºC temperature and 210 - 300 µmol/m2•s light intensity (Department of Agricultural, Food and Nutritional Science, University of Alberta, AB, Canada) using the RNeasy Mini Kit (Qiagen, Maryland, USA). On-column DNA digestion was

carried out to remove traces of DNA in RNA samples using the On-Column RNase-Free DNase Set (Qiagen). First-strand cDNA synthesis was carried out using the Superscript III first-strand cDNA synthesis kit according to the manufacturer's instructions using oligodT primers (Invitrogen, Life Technologies Inc.) with 350 ng RNA used as template.

3.2.3 Amplification of *AtCESA1* and *AtCESA9* cDNA fragments

The desired double-stranded cDNA sequences were obtained through PCR amplification using primers shown in Table 3.1. Primers were designed to produce a 320-bp fragment and 371-bp fragment for *AtCESA1* and *AtCESA9*, respectively. These fragments were subsequently used for developing RNAi cassettes to down-regulate *AtCESA1* in a seed-specific manner and *AtCESA9* both seed-specifically and constitutively. Each primer contains restriction sites near their 5' ends (indicated in bold), to facilitate cloning.

PCR amplification was conducted using the high fidelity Pfx50 PCR reaction system (Invitrogen). Each PCR reaction mixture consisted of 2µL of cDNA, 2 µl of Pfx50 PCR mix, 0.75µl of dNTPs (10mM each), 0.75 µl of each of the forward and reverse primers (10µM), 1 µl of Pfx50 DNA polymerase and 17.75 µl of nuclease-free water. Thermal parameters for amplification were 94ºC for 2 min, 40 cycles of 94ºC for 15s, 60ºC for 30s, and 68ºC for 1.0 min, followed by a final extension at 68ºC for 5 min. Amplicons were electrophoresed on a 1% agarose gel for visualization of the amplified products. The visualized bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen).

Table 3.1 Primers for amplification of *AtCESA1* and *AtCESA9* fragments

Gene	Primer name	Primers	Fragments used for
AtCESA1	Forward1- CESA1RNAi**NcoI**	5'-**CCATGG**TGCAGGATGGTACTCCCTGGG-3'	
	Reverse1- CESA1RNAi**PstI**	5'-**CTGCAG**CAGCACTTCTTTCCAATAGC-3'	seed specific construct
	Forward 2 - CESA1RNAi**EcoRI**	5'-**GAATTC**TGCAGGATGGTACTCCCTGG-3'	
	Reverse 2 - CESA1RNAi**HindIII**	5'-**AAGCTT**CAGCACTTCTTTCCAATAGC-3'	
ATCESA9	Forward1- CsRNAi**NcoI**	5'-**CCATGG**TTCTCATTAACGCCGACGAC-3'	
	Reverse1- CsRNAi**PstI**	5'-**CTGCAG**ACACTTCATCAGTACCACG-3'	seed specific construct
	Forward2- CsRNAi**EcoRI**	5'-**GAATTC**GTTCTCATTAACGCCGACGAC-3'	
	Reverse2-CsRNAi**SmaI**	5'-**CCCGGG**ACACTTCATCAGTACCACG-3'	
ATCESA9	Forward1-CESA9RNAi**SalI**	5'-**GTCGAC**GTTCTCATTAACGCCGACGAC-3'	
	Reverse1-CESA9RNAi**EcoRI**	5'-**GAATTC**GACACTTCATCAGTACCACG-3'	constitutive construct
	Forward2- CESA9RNAi**XbaI**	5'-**TCTAGA**GTTCTCATTAACGCCGACGAC-3'	
	Reverse2-CsRNAi**SmaI**	5'-**CCCGGG**ACACTTCATCAGTACCACG-3'	

3.2.4 Cloning *AtCESA* cDNAs into the pGEM®-T Easy vector (TA cloning)

A poly deoxy-adenosine (A) overhang was added to the 3´ ends of the PCR products to facilitate subsequent TA cloning by mixing 25.3 µl of each purified PCR product, 3 µl of 10× PCR buffer minus Mg^{2+}, 0.6 µl of dATP (10 mM), 0.9 µl of $MgCl_2$ (50 mM) and 0.2 µl of Taq DNA polymerase, and incubating at 72ºC for 20 min. The resulting cDNA fragments were then cloned into pGEM®-T Easy vectors (Promega, Madison, USA) and introduced into MAX Efficiency® DH5α™ Competent *Escherichia coli* cells (Invitrogen, Allen Way Carlsbad, Canada) according to the manufacturer's recommendations. Subsequently, 75µl of the transformation mixtures were plated on 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (18 µl) that had been spread onto Lysogeny broth (LB)/ ampicillin (100 mg/ml) plates. Positive white colonies were selected and single colonies were transferred into LB/ampicillin (100 mg/L) medium consisting of 1% [w/v] tryptone or peptone, 0.5% [w/v] yeast extract, 0.5% NaCl, in water. Plasmids were isolated using the GeneJET TM Plasmid Minprep Kit (Thermo Fisher Scientific) from overnight cultures and the presence of expected DNA in the colonies was confirmed by

restriction digestion with EcoRI (FastDigest; Thermo Fisher Scientific) according to the manufacturer's recommendations. The sequences of the cloned *CESA* fragments were confirmed by DNA sequencing using M13F- primers (Molecular Biology Facility, Department of Biological Sciences, University of Alberta, AB, Canada).

3.3 Generation of *AtCESA* RNAi plant transformation vectors

3.3.1 Generation of *AtCESA* RNAi auxiliary vectors

Three different RNAi cassettes were developed, including an *AtCESA1* seed-specific RNAi cassette, an *AtCESA9* seed-specific RNAi cassette, and an *AtCESA9* constitutive RNAi cassette. The first step was the introduction of the RNAi fragments into the auxiliary pAUX3131 vector (Wesley et al., 2001) that had been previously modified to contain either a seed-specific or constitutive promoter, along with an intronic spacer and transcriptional terminator (modified by S. Singer). Subsequently, the plasmids were transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen) and plasmids were isolated. Sequencing of the resulting plasmids confirmed that the *AtCESA9* RNAi fragments were inserted in opposite orientations between the constitutive tCUP3 promoter and intronic spacer from the pKannibal plasmid (Wesley et al., 2001), and ribulose-1,5-bisphosphate carboxylase/oxygenase terminator (rbcS-t) transcriptional terminator and intronic spacer, respectively, in the case of the *AtCESA9* constitutive RNAi cassette. These same sense and antisense cDNA fragments were also inserted between the seed-specific *Phaseolus vulgaris* β-Phaseolin promoter (Frisch et al., 1995) and pKannibal intronic spacer, and between the β-Phaseolin transcriptional terminator and intronic spacer, respectively, in the case of the *AtCESA9* seed-specific RNAi construct. In the case of the *AtCESA1* seed-specific RNAi construct, *AtCESA1* RNAi fragments were inserted in opposite orientations between the seed-specific *P. vulgaris* β-Phaseolin promoter and pKannibal intronic spacer, and

the β-Phaseolin transcriptional terminator and intronic spacer, respectively. After each ligation step, test restriction digestions were conducted and sequences were verified by DNA sequencing with both promoter-specific and cDNA fragment-specific primers.

3.3.2 Cloning of RNAi cassettes into binary vector systems

The resulting RNAi cassettes were then introduced into a modified plant transformation vector derived from pPZP-RCS1 (Goderis et al., 2002), which contained a kanamycin-resistance gene, *Neomycin phosphotransferase II* (*NPTII*) under the control of the *Nopaline synthase* (*nos*) promoter (Figure 3.1). To generate the seed-specific *AtCESA1* and *AtCESA9* RNAi plant transformation vectors, the corresponding cassettes were excised from their auxiliary vectors using Asc1 endonuclease, and then separated on a 1% agarose gel along with pPZP-RCS1 vector digested with same endonuclease. This step was followed by purification of the desired digested products using the QIAquick Gel Extraction kit (Qiagen). The digested pPZP-RCSI backbones were treated with FastAP thermosensitive alkaline phosphatase (Thermo Fisher Scientific) before ligation with the digested RNAi expression cassettes. To produce the *AtCESA9* constitutive RNAi plant transformation vector, the corresponding cassette and pPZP-RCSI backbone were digested with PI-PspI endonuclease, followed by the same protocol as described above (Figure 3.1). The resulting RNAi plasmids were introduced into chemically competent *E. coli* cells (Invitrogen) and extracted plasmids were subjected to sequence verification using DNA sequencing with promoter-specific and cDNA fragment-specific primers.

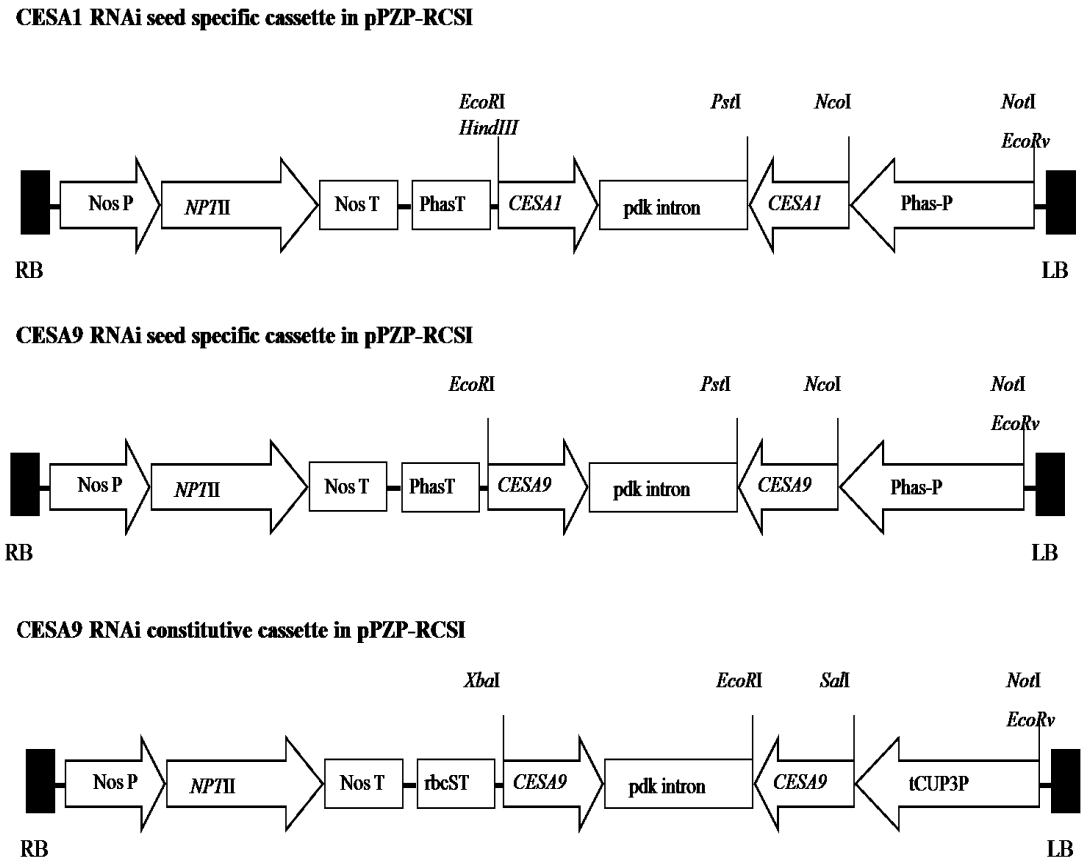

Figure 3.1 Schematic representations of *AtCESA* binary vectors developed for plant transformation

Arrows indicate the direction of transcription in each case. *CESA1* and *CESA9*, Arabidopsis *AtCESA* sequence; LB, left T-DNA border; NosP, *Nopaline synthase* promoter; NosT, *Nopaline synthase* transcriptional terminator; NPTII, *Neomycin phosphotransferase II*; Phas-P, *Phaseolus vulgaris Phaseolin* promoter; PhasT, *Phaseolin* transcriptional terminator; RB, right T-DNA border; rbcST, *Pisum sativum Ribulose-1,5-bisphosphate carboxylase* transcriptional terminator; tCUP3P, tobacco *tCUP3* constitutive promoter.

3.4 Agrobacterium-mediated Arabidopsis plant transformation

The RNAi vectors were introduced into Arabidopsis ecotype Col-0 wild-type plants using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998) as described in the following sub sections.

3.4.1 Preparing Electro-competent *Agrobacterium tumefaciens* cells

Electro-competent *Agrobacterium tumefaciens* cells (GV3101 strain) were prepared based on a protocol described in Clough and Bent (1998). Agrobacterium cells were allowed to grow in 5 ml of LB medium containing gentamycin (25 µg/ml) and rifampicin (50 µg/ml) for 16 h at 28℃ (220 rpm). One mL of culture was then transferred to 10 mL of fresh LB medium. After incubation for 16 h (28℃, 220 rpm), 800 µl was transferred to 50 ml of fresh LB medium and cells were cultured until the OD600 reached 0.5 (approximately 7 hrs). The resulting cells were then pelleted by centrifugation (3,000 x g, 15 min at 4℃), at which point they were washed twice with ice-cold sterile water (40 ml) and twice in sterile ice-cold 10% glycerol (40 ml). Finally, the cells were suspended in 0.5 ml of sterile ice-cold 10% glycerol and aliquoted (40 µl) into Eppendorf tubes and stored at -80℃ until further use.

3.4.2 Introduction of RNAi vectors into *Agrobacterium tumefaciens*

RNAi vectors were introduced into Agrobacterium cells using electroporation. Specifically, 40 µl of competent Agrobacterium cells were mixed with 0.5 µl of each pPZP-RCS1-derived RNAi vector and empty vector control, respectively, in sterile Eppendorf tubes and incubated for 10 minutes on ice. The mixtures were then gently transferred to ice-cold Gene Pulser Cuvettes (Bio-Rad, Mississauga, Ontario) and electroporated (1.8 volts, 1 s) using a MicroPulser™

Electroporator (Bio-Rad). Transformed cells were incubated at 28ºC for 3h (220 rpm) in 1 ml of LB medium containing spectinomycin (50 µg/ml) and gentamycin (30 µg/ml). The resulting Agrobacterium cultures (50 µl) were then plated on LB agar media plates (containing 50 µg/ml spectinomycin and 30 µg/ml gentamycin) at 28℃ for 2 days. Five ml of LB broth (containing 50µg/ml spectinomycin and 30 µg/ml gentamycin) was inoculated with transformed colonies and incubated at 28℃ for 16h. 50% glycerol stocks were prepared using 700 µl of overnight bacterial cultures and stored at -80℃ until further use.

3.4.3 Transformation of Arabidopsis using the floral dip method

LB broth cultures (10 ml) containing 50 µg/ml spectinomycin and 30 µg/ml gentamycin inoculated with pPZP-RCS1 derived RNAi vectors, along with the empty vector control, were incubated (28℃, 220 rpm) for 24 h. These cultures were introduced into 300 ml of LB broth containing spectinomycin (50 µg/ml) and gentamycin (30 µg/ml), followed by incubation at 28ºC for 24 h (220 rpm). Agrobacterium cells containing each RNAi vector and the empty pPZP-RCS1 vector, respectively, were pelleted by centrifugation (6000 rpm at 20℃ for 10 min). The cell pellets were re-suspended in 400 ml of freshly prepared infiltration medium (5% sucrose solution containing 0.05% (v/v) silwet-L77). Arabidopsis plants (8-10 plants per ½ L plastic pot, 5 pots per construct) were inverted and dipped into the suspensions for approximately 30 seconds. Subsequently, plants were covered with plastic domes for 24 h to maintain humidity, and were then allowed to grow in a growth chamber as previously described. Seeds (T_1) were harvested when siliques had matured and dried.

3.5 Screening of transgenic plants

After harvesting the T_1 seeds, the plants were screened for the presence of transgene as follows. First, seeds were surface sterilized by soaking in 70% (v/v) ethanol for 30 s, then in 30% (v/v) bleach containing Triton X-100 for 20 min, and were subsequently washed five times with sterile water. Sterilized seeds were then spread on selection medium (Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (0.21% [w/v] MS basal salts, 1% [w/v] sucrose, 0.03% [w/v] MES, 0.8% [w/v] phytagar, 20 mg/ml kanamycin, 99.6 µg/ml timentin, pH 5.7-5.8). Plates were incubated in the dark at 4℃ for 2-3 days and then transferred to a growth room at 23 ºC with a 250 µmol/m2•s light intensity for 7-10 days. Kanamycin resistant plants (36 from each construct) were transferred to soil individually and allowed to grow in a growth chamber until they produced T_2 seeds.

T_2 seeds were harvested and cleaned when they had matured and dried. Seeds were then subjected to surface sterilization as above followed by planting on screening media individually using the same protocol as above. Plants bearing a single copy of the transgene were identified by selecting lines which yielded an approximately 3:1 ratio of kanamycin-resistant to non-resistant plants. Kanamycin-resistant single copy plants were grown individually to obtain T_3 seeds.

T_3 seeds from each single T_2 plant were also screened on kanamycin-selection plates to select homozygotes and heterozygotes. Plants which produced only kanamycin resistant seeds were selected as homozygous lines whereas lines with a kanamycin resistant to non-resistant ratio close to 3:1 were considered to be heterozygous.

3.6 Determination of Arabidopsis total lipid content and fatty acid composition

Approximately 10 mg of cleaned, dried T_2 and T_3 mature seeds, respectively, were weighed and placed in hexane washed Teflon-lined screw capped glass tubes. C17:0 TAG (100 µl) internal standard was dispensed onto the bottom of the tube and dried under nitrogen gas. Two ml of 3N methanolic HCl (Supelco, Sigma-Aldrich, Oakville, Ontario) was added and the tubes were incubated for 16 h at 80℃ for transmethylation to occur. Subsequently, the tubes were cooled on ice for 5 min and 2 ml of 0.9% NaCl was added to stop the reaction. The resulting fatty acid methyl esters (FAMEs) were extracted twice by adding 2 ml of hexane, vortexing for 30 s, centrifuging at 2,000 x g for 4 min, and transferring the hexane phases (upper layer) to a fresh glass tube. The hexane extracts were pooled and dried under nitrogen gas at 37℃ until all hexane had evaporated. The resulting FAMES were then redissolved in 1 ml of iso-octane containing C21:0 methyl ester (0.1 mg/ml) and analyzed by gas chromatography (GC)-mass spectrometry (MS).

For GC analysis (Agilent Technologies 7890A GC system), a split/splitless inlet was used and the injection volume was 1 µl in the ten-to-one split mode; FAME separation was performed in a DB-23 capillary column (Agilent Technologies: 30 m × 250 µm × 0.25 µm) with helium as the carrier gas (1.2 ml/min). The temperature program was as follows: 165℃ for 4 min, 165-180℃ for 5 min, and 180-230℃ for 5 min. Ingredients were detected by mass spectrometry (Agilent Technology 5977A Mass Selective Detector) and peaks were identified with the software NIST MS Search 2.0.

3.7 Total protein content analysis

Total protein content was analyzed in T_3 homozygous seeds using a modified "Dumas" method (AOAC 992.23). Briefly, the nitrogen content was estimated in duplicate T_3 homozygous dry seed samples (80-105 mg) using a nitrogen analyzer (model FP-428, Leco Instruments Ltd., Mississauga, ON, Canada). The total protein content in the seed samples were then determined by multiplying the nitrogen content by a conversion factor of 6.25 (AOAC 992.23). The total Caffeine (157 mg) and EDTA (Ethylenediaminetetraacetic acid; 102 mg) contents were used as standards for calibration.

3.8 Crystalline cellulose content analysis

Crystalline cellulose content of T_3 homozygous seed samples was determined using a microscale method based on that of Updegraff (1969) with slight modifications (Griffiths et al., 2014). Ten to 20 mg of seed were ground into a powder in liquid nitrogen using a mortar and pestle followed by incubation at 50ºC for 16 h after washing with 70% ethanol. The exact dry weights of the homogenized seeds were recorded, and samples were then treated with 2 ml of Updegraff reagent (acetic acid: nitric acid: water, 8:1:2 (v/v)). Samples were mixed well by vortexing and incubated at 100ºC for 1 h, followed by centrifugation at 13000 ×g for 5 min. Pellets were washed with miliQ water and acetone (×2), and then incubated at room temperature overnight until the samples were completely dry. Samples were then treated with 1 ml 72% (w/v) H_2SO_4, vortexed, incubated at room temperature for 90 min, followed by 5 min centrifugation at 10,000 ×g. Samples were then diluted 10 times in distilled water. The glucose content of the samples was measured using a colorimetric method in microtiter plates (Foster et al., 2010). Briefly, 75 µl of diluted sample was treated with 150 µl of freshly prepared cold (4°C) anthrone reagent (2mg/ml

anthrone [Sigma-Aldrich] in concentrated H_2SO_4) in microtiter plates. The anthrone mixtures were then incubated for 30 min at 80°C, and absorbance was recorded at a wavelength of 620 nm in a spectrophotometer (Synergy H4 Hybrid reader, Biotek instruments, Winooski, USA). A standard curve was prepared from a standard serial dilution of glucose, and total amounts of cellulose-derived glucose were calculated per weight of dry seed mass.

3.9 Seed weight analysis

Approximately 150-250 T_3 homozygous seeds were counted in triplicate using the particle counter function of the FuorChem SP Imager and AlphaEase software (Alpha Innotech Corp., San Leandro, CA), followed by weighing using an analytical balance (OHAUS Corporation, Florham Park, NJ).

3.10 Quantitative real-time RT-PCR

3.10.1 Total RNA extraction and cDNA synthesis

Total RNA was extracted from T_2 and homozygous T_3 developing siliques using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). On-column DNA digestion was carried out to remove traces of DNA in RNA samples using the On-Column DNase I Digest Set (Sigma-Aldrich). First-strand cDNA synthesis was carried out using the Superscript IV first-strand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen, Life Technologies Inc.). Briefly, 1 µl of oligo dT primer (0.5 µg/µl), 1 µl of dNTPs (20 mM) and 0.5 µl of RNase free water were added to 350 ng of DNase-treated RNA and incubated at 65ºC for 5 min followed by snap chilling on ice. Subsequently, 4 µl of 5X first-strand buffer, 0.5 µl of RNase Out and 2 µl of dithiothreitol (DTT) (0.1 mM) were added and incubated at 42ºC for 2 min. After adding 1 µl of Superscript IV, the reaction mixtures were incubated at 42ºC for 50 min and 70ºC for 15 min in a

thermocycler (GeneAmp PCR system, AB applied Biosystems, Singapore). Samples were stored at -20 ^{0}C until further use.

3.10.2 Expression analysis of *AtCESA1* and *AtCESA9*

Quantitative RT-PCR assays were performed in triplicate using 1 µl of a 1/5 diluted cDNA as template along with SYBR green PCR master mix in a total reaction volume of 10 µl according to the manufacturer's instructions (Applied Biosystems, Life Technologies Inc.). *AtCESA1* expression was analyzed in seed-specific *AtCESA1* down-regulated lines along with control lines using *AtCESA1* gene-specific primers (Table 3.2). *AtCESA9* expression analysis was carried out in both seed-specific and constitutive *AtCESA9* down-regulated lines along with control lines using *AtCESA9* gene-specific primers (Table 3.2). Constitutively expressed *PROTEIN PHOSPHATASE 2A SUBUNIT 3* (*PP2AA3*) transcript was used as the internal control, and it was amplified using gene-specific primers (Table 3.2) (Singer et al., 2016). Assays were conducted using a Step OnePlus, Realtime PCR System (Life Technologies) using the following thermal parameters, 95ºC for 2 min, followed by 40 cycles of 95ºC for 15s and 60ºC for 1 min. Levels of gene expression were obtained using the comparative Ct method (Schmittgen and Livak, 2008).

Table 3.2 Primers used for expression analysis of *AtCESA* genes

Target genes/ Reference gene	Primers	Amplicon length (nt)
AtCESA1 (Target gene)	F: CATGAAGGGAAAGGAGGAGAAA R: GTTAGGCGAGAAGATGGGATAG	119
AtCESA9 (Target gene)	F: GAAGGGAAGCCATCAGAGTTAG R: GCGAGAATAGACAGAACCGTATT	107
PP2AA3 (Reference gene)	F: TCAATCCGTGAAGCTGCTGCAAAC R: ACTGCACGAAGAATCGTCATCCGA	146

3.11 Statistical analysis

Probability values (P-values) were calculated using a one-tailed student's T test (assuming unequal variance) for the comparisons of the empty vector control line with the AtCESA RNAi lines for seed weight (Figure 4.4), oil content (Figure 4.5 and 4.6), FA composition (Table 4.1), protein content (Figure 4.7) and cellulose content (Figure4.8). Statistical significance was declared at $P \leq 0.05$ for comparisons between plant lines.

4. Results

4.1 Validation of seed-specific and constitutive *CESA* RNAi Arabidopsis lines

Seed-specific *AtCESA1* RNAi, *AtCESA9* seed-specific and constitutive RNAi constructs were developed (Figure 3.1) in order to down-regulate the expression of these genes and determine their effects on seed oil, protein and cellulose contents. Initial expression analysis to confirm the degree of down-regulation of *AtCESA1* and *AtCESA9* genes was performed on T_1 siliques containing T_2 seeds (14 days after flowering [DAF]). As shown in Figure 4.1A, the relative expression level of the *AtCESA1* gene was reduced up to 79% in the *AtCESA1* RNAi lines (CESA1ss-3) compared to control lines (Con). A high variability in the degree of down-regulation of the *AtCESA9* gene was observed among the tested Arabidopsis lines (Figure 4.1B). The highest reduction of *AtCESA9* gene expression was 66% and 70% for CESA9ss-2 and CESA9c-8 lines, respectively. Lines CESA9ss-8 or CESA9c-1 did not exhibit a high degree of *AtCESA9* gene down-regulation. Furthermore, developing T_2 siliques containing T_3 seeds (14 DAF) were also harvested from independent *AtCESA1* and *AtCESA9*-RNAi lines (utilized throughout this study), respectively, followed by the identification of homozygous lines in each case. Appropriate alterations in *AtCESA* expression were also confirmed in this tissue type (Figure 4.2). In *AtCESA1* RNAi T_3 siliques, the relative expression level of *AtCESA1* was reduced up to 68% (CESA1ss4-13) compared to levels observed in control lines (Con). T_3 seeds harvested from *AtCESA9* T_2 RNAi lines also showed relatively low expression of the *AtCESA9* gene compared to control lines. There was still some variability, however, in the expression levels of *AtCESA9* in down-regulated lines. Compared to control lines, the highest reductions observed for *AtCESA9* gene expression were 55% and 36% in *AtCESA9* seed-specific (CESA9ss6-4) and *AtCESA9* constitutive (CESA9c8-4) lines, respectively.

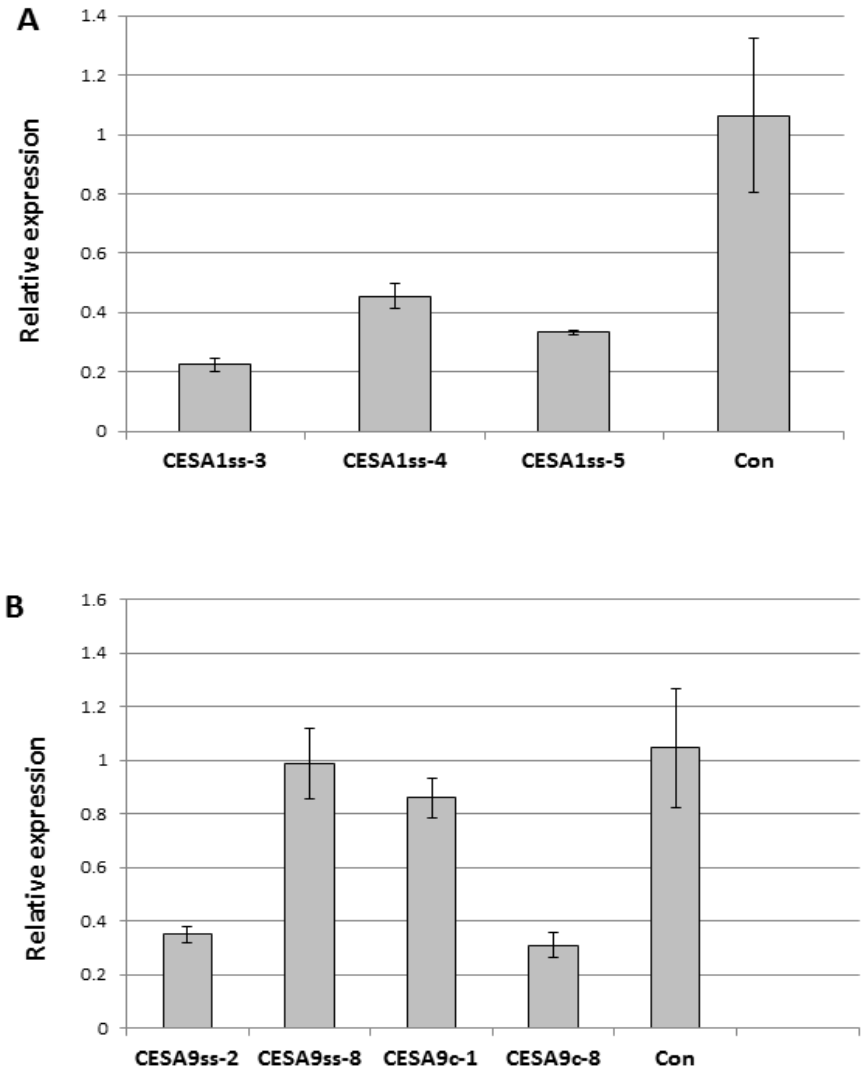

Figure 4.1 Relative expression of Arabidopsis *AtCESA1* (A) and *AtCESA9* (B) genes. Total RNA was obtained from T_2 Arabidopsis seeds at 14 days after flowering. Arabidopsis seeds transformed with RNAi cassettes driven by the ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and under the control of constitutive tCUP promoter for *AtCESA9* gene (CES9c). Controls (Con) are the plants transformed with empty vector. Equal amounts of total RNA were used for cDNA synthesis and serial dilutions of the resulting reactions were used for quantitative RT-PCR. Each bar represents the mean from three determinations ± SE with the Arabidopsis *PROTEIN PHOSPHATASE 2A SUBUNIT 3* (*PP2AA3*) gene as the internal reference gene. (Con) bar represents the mean from 3 individual plant lines with 3 technical repeats for each plant.

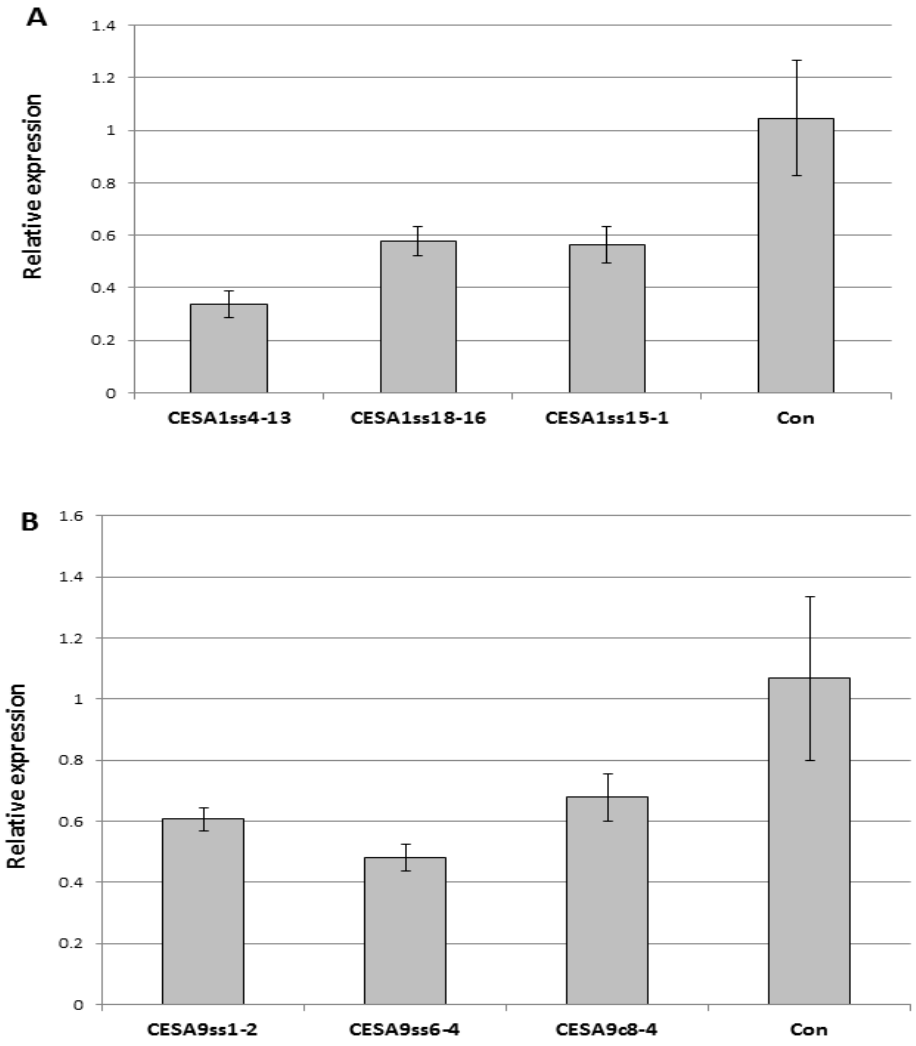

Figure 4.2 Relative expression of Arabidopsis *AtCESA1* (A) and *AtCESA9* (B) genes. Total RNA was obtained from T_3 homozygous Arabidopsis seeds at 14 days after flowering. Arabidopsis seeds transformed with RNAi cassettes driven by ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and under the control of constitutive tCUP promoter for *AtCESA9* gene (CES9c). Controls (Con) are the plants transformed with empty vector. Equal amounts of total RNA were used for cDNA synthesis and serial dilutions of the resulting reaction were used for quantitative RT-PCR. Each bar represents the mean from three determinations ± SE with the Arabidopsis *PROTEIN PHOSPHATASE 2A SUBUNIT 3* (*PP2AA3*) gene as the internal reference gene. (Con) bar represents the mean from 3 individual plant lines with 3 technical repeats for each plant.

4.2 Seed-specific down-regulation of *AtCESA1* and constitutive down- regulation of *AtCESA9* did not affect Arabidopsis vegetative tissue growth or T_3 seed morphology and weight

There were no apparent visual phenotypic defects (in seeds or plants) (Figure 4.3) in any of the generations of transgenic lines studied except for a minor effect on root growth at the very beginning stages of seed germination (data not shown). The seeds of *AtCESA1* and *AtCESA9* down-regulated lines were further analyzed for seed weight. Batches of 130- 200 seeds (in duplicate) from each seed sample were weighed and expressed as weight for 100 seeds. Seed weight data are presented in Figure 4.4. Neither seed-specific down-regulation of *AtCESA1* nor constitutive down-regulation of *AtCESA9* genes significantly affected 100-seed weight. Seed-specific down-regulation of *AtCESA9*, however, resulted in a 7% relative reduction in seed weight compared to control lines (P value 0.006). On average, 100-seed weight was reduced from about 2.18 mg (lowest 1.93 mg; highest 2.48 mg) for the control lines to 2.02 mg (lowest 1.76 mg; highest 2.23 mg) for *AtCESA9* seed-specific RNAi lines. Furthermore, CESA9ss-6 or CESA9ss-13 showed 8% or 10.4% reduction, respectively, in relative seed weights compared to those of control lines.

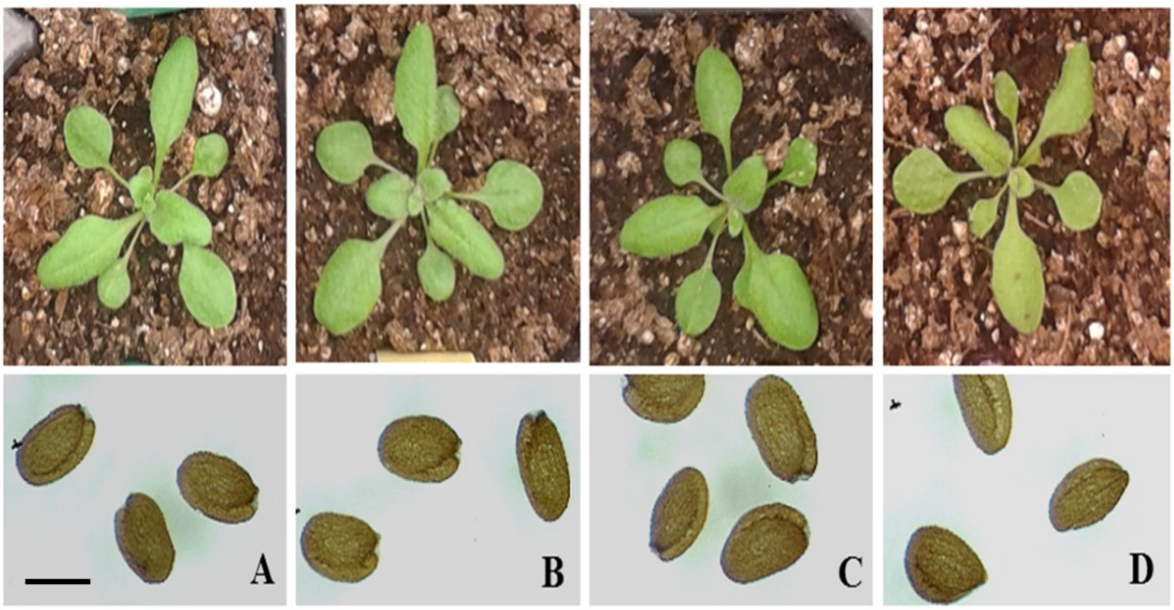

Figure 4.3 *AtCESA* RNAi plants and seed morphology compared to empty vector control lines. There were no apparent phenotypic differences observed in any of RNAi lines compared to wild type Arabidopsis plants. The upper row of images represents 18 day old soil grown plants and the lower row of images represents homozygous mature dry seeds as observed using a dissecting light microscope. Scale bar represents 200 µm. **(A)** Empty vector control **(B)** *AtCESA1* seed-specific RNAi **(C)** *AtCESA9* seed-specific RNAi **(D)** *AtCESA9* constitutive RNAi.

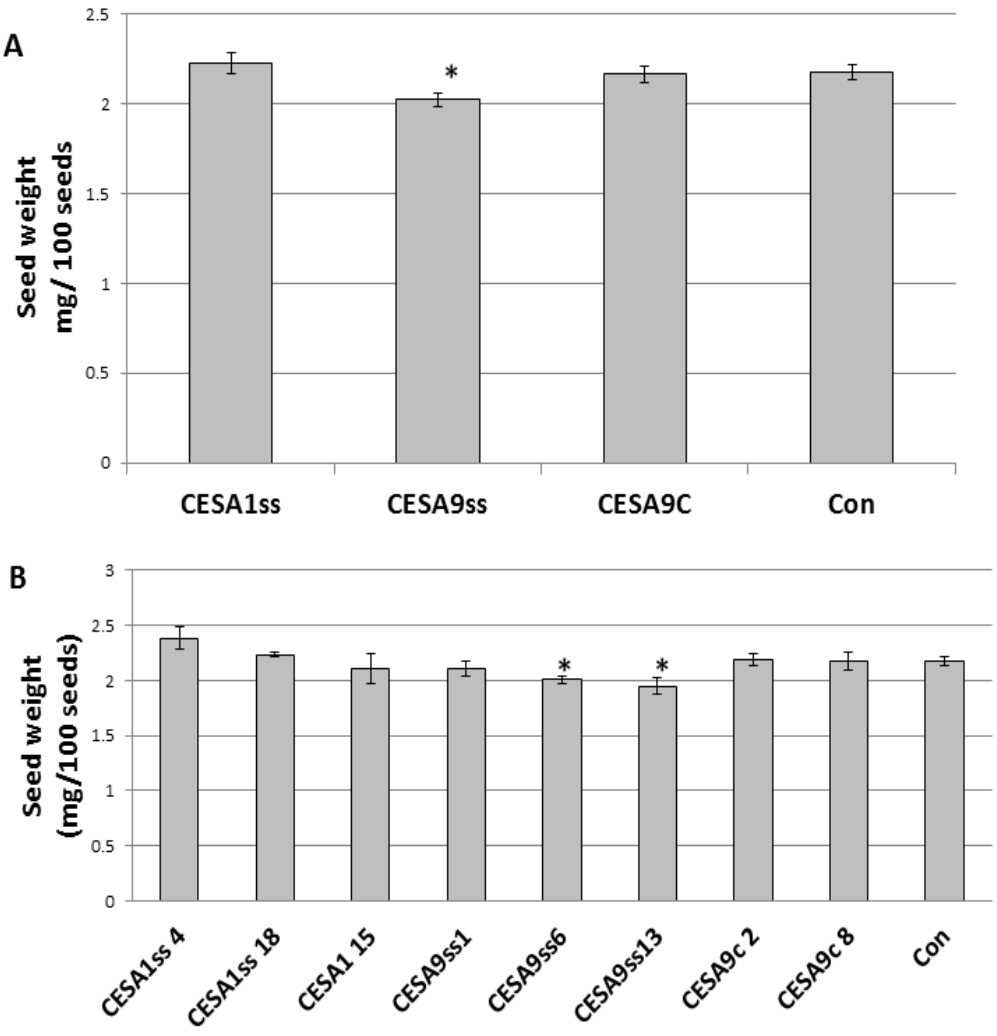

Figure 4.4 One hundred-seed weight for T_3 homozygous Arabidopsis seeds transformed with RNAi cassettes driven by ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and driven by constitutive tCUP promoter for *AtCESA9* gene (CESA9c). **(A)** Each bar represents the average of 7-14 transgenic T_3 seed samples coming from plant lines transformed with corresponding construct. **(B)** Each bar represents the average of 3-4 transgenic T_3 seed samples coming from plant lines which have single copy DNA insertion in the same locus. In both graphs, error bars represents the standard error of the mean. Controls (Con) are the plants transformed with empty vector (In Both A and B represent the average of 6-11 biological repeats). A significant difference in seed weight compared to the control is marked with *[$P \leq 0.05$; in one-tailed student's T-test]

4.3 Down-regulation of *AtCESA* genes did not increase total seed oil content and had only minor effect on the fatty composition of the seed oil

4.3.1 Total seed oil content of T_2 and T_3 Arabidopsis seeds

Initially, T_2 seeds from 10-20 independent transgenic lines for each construct were harvested and subjected to total oil content analysis to determine the effect of *AtCESA* down-regulation on seed oil biosynthesis. There was tendency towards reduced seed oil content in T_2 seeds with seed-specific down-regulation of the *AtCESA1* gene. As shown in Figure 4.5, there was ~1.5% (P value, 0.001) reduction in seed oil content compared to the empty vector control in those lines. On average, seed oil content was 29.54% (w/w) for the control line and up to 27.98 % for the *AtCESA1* RNAi line. The change in seed oil content, however, was relatively small. The constitutive down-regulation of *AtCESA9* did not affect seed oil content in T_2 Arabidopsis seeds significantly (oil content, 29.3%). *AtCESA9* seed-specific down-regulation also showed ~1% reduction (P value, 0.04) of seed oil content in T_2 seeds. Total seed oil content ranged from 28.2% - 31.3% in control lines, 26.2% -30.7% in *AtCESA1*seed-specific RNAi lines, 26.9%-30.1% in *AtCESA9* seed-specific RNAi lines and 26.2%-31.7% in *AtCESA9* constitutive RNAi lines.

The transgenic lines were further propagated to obtain T_3 seeds representing homozygous plants having a single insertion. More specifically, T_2 seeds were subjected to segregation analysis and three T_1 plant lines were selected from each type of RNAi line (*AtCESA1* seed-specific, *AtCESA9* seed-specific, *AtCESA9* constitutive) which exhibited a 3:1 ratio of kanamycin-resistance to non-resistance as single insertion of transgene containing T_1 lines. Thereafter, 18 plants (T_2) from each selected single transgene copy inserted transgenic line were grown to obtain T_3 seeds. T_3 seeds were subjected to segregation analysis in order to select

homozygotes (three to four lines from each family) which were analyzed for seed oil content. In the T_3 generation, the average oil content data reflected the previous T_2 seed oil content data with lower total oil content observed in *AtCESA1* and *AtCESA9* seed-specifically down-regulated lines, compared to the empty vector control (Figure 4.6A). On average, the seed oil content was about 29.0% (highest; 30.33% lowest 27.25%) for the control lines and 28.2 % (highest 29.36%; lowest 26.86 %; P value, 0.01) and 28.3% (highest 30.32%; lowest 27.08%; P value 0.03), respectively, for *AtCESA1* and *AtCESA9* seed-specific RNAi lines. The reduction in seed oil content, however, was less than one percentage point for both *AtCESA1* and *AtCESA9* RNAi lines compared to empty vector control lines. *AtCESA9* constitutive down-regulation also resulted in a minor reduction in seed oil content (to 28.4%) in T_3 Arabidopsis seeds.

In further analysis, average seed oil content data were calculated for homozygotes coming from the same T_2 seed lines with insertion at one locus in the Arabidopsis genome (isogenic lines) (Figure 4.6B). This analysis revealed that two of these isogenic lines from *AtCESA1* seed-specific RNAi lines (CESA1ss1; P value 0.02 and CESA1ss4; P value, 0.03) and one of the lines from *AtCESA9* seed-specific RNAi lines (CESA9ss1; P value 0.004) exhibited a tendency for reduced seed oil content (Figure 4.6B). None of the isogenic lines carrying *AtCESA9* constitutive constructs showed significant changes in seed oil content (Figure 4.6B). Collectively, these data suggest that total seed oil content was not increased in both *AtCESA1* and *AtCESA9* down-regulated RNAi lines. There was, however, an apparent minor reduction in seed oil content in both *AtCESA1* and *AtCESA9* RNAi lines.

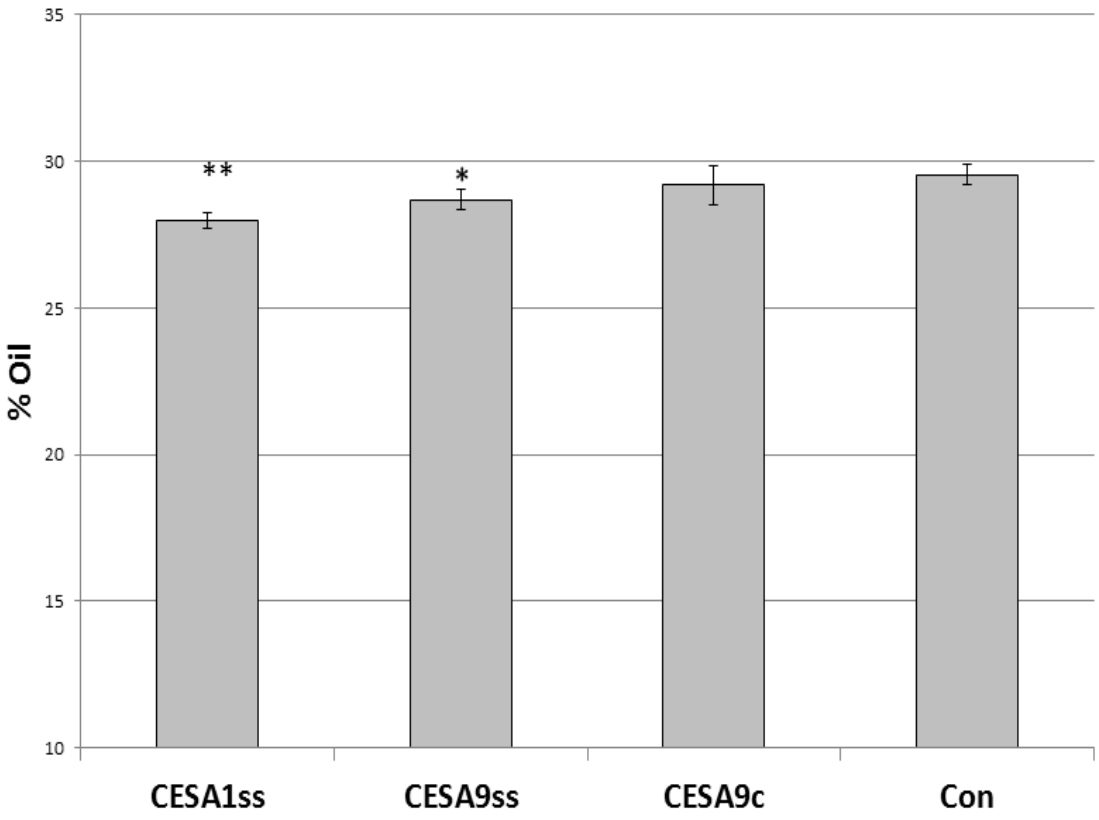

Figure 4.5 Seed oil content as percentage of dry seed weight from T_2 Arabidopsis seeds transformed with RNAi cassettes driven by ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and under the control of constitutive tCUP promoter for *AtCESA9* gene (CES9c). Each bar represents the average of 10-20 independent transgenic lines transformed with corresponding construct. The error bars represent standard error of the mean. Controls (Con) are the plants transformed with empty vector. A significant difference in oil content compared to the control is marked with *($P \leq 0.05$) and very significant with **($P \leq 0.01$ in one-tailed student's t-test.

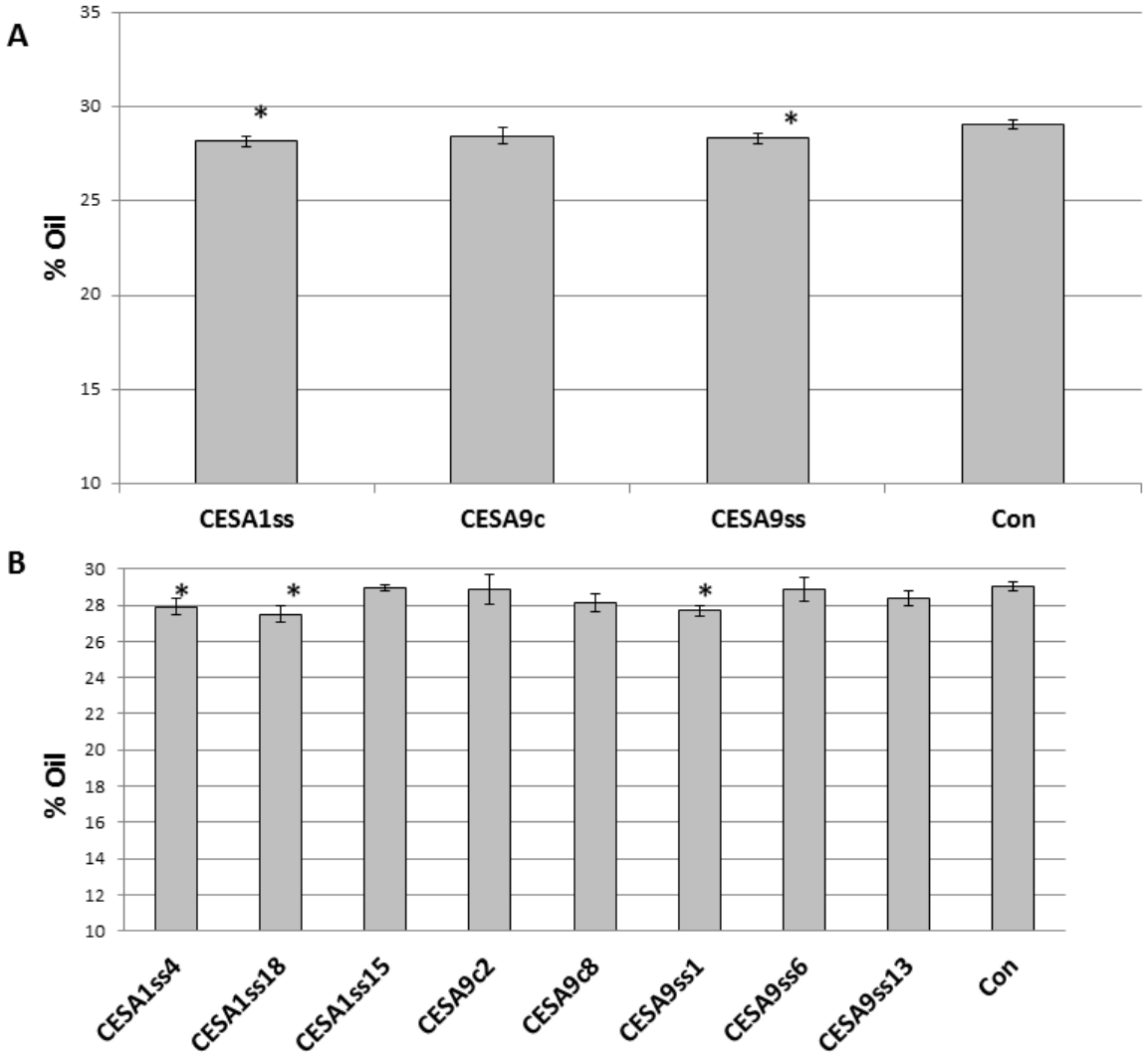

Figure 4.6 Seed oil content as percentage of dry seed weight from T_3 homozygous Arabidopsis seeds transformed with RNAi cassettes driven by ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and under the control of constitutive tCUP promoter for *AtCESA9* gene (CES9c). **(A)** Each bar represents the average of 7-14 transgenic T_3 seed samples coming from plant lines transformed with corresponding construct. **(B)** Each bar represents the average of 3-4 transgenic T_3 seed samples coming from plant lines which have single copy DNA insertion in the same locus. In both graphs, error bars represents the standard error of the mean. Controls (Con) are the plants transformed with empty vector (In Both A and B represent the average of 6-11 biological repeats). A significant difference in seed oil content compared to the control is marked with *[$P \leq 0.05$; in one-tailed student's T-test]

4.3.2 Fatty acid composition of the seed oil of *AtCESA1* and *AtCESA9* RNAi lines

To determine whether *AtCESA1* or *AtCESA9* down-regulation affected FA composition, FA compositional analysis was conducted for the oil extracted from T_3 homozygous seeds. *AtCESA1* or *AtCESA9* seed-specific down-regulation had only very minor effects on FA composition. There was some increase in the average proportions of C16:1Δ^{9cis}(hereafter C16:1) in seed oil obtained from *AtCESA1* andr *AtCESA9* seed-specific RNAi lines with P values of 0.01 and 0.03 in student's T-test respectively. The C16:1 composition was 0.39% in both *AtCESA1* and *AtCESA9* seed-specific T_3 seeds compared to 0.36% in control lines. The proportion of C16:1in *AtCESA1* seed-specific lines ranged from 0.34%- 0.42% and in *AtCESA9* seed-specific lines ranged from 0.36% - 0.51%, with 0.34% - 0.39% in control lines (Table 4.1). Furthermore, C16:0 content exhibited some enhancement in *AtCESA1* or *AtCESA9* seed-specific down-regulated T_3 seeds. The mean C16:0 content of oil samples from control lines was 8.6% (lowest 8.38%; highest 8.86%), while most of the samples ranged from 8.4% to 8.7% C16:0. In contrast, the mean of C16:0 content in samples of *AtCESA9* seed-specific RNAi lines was 8.85%, which was somewhat higher compared to control lines (P value, $1.8X10^{-5}$). The C16:0 content of the seed oil of *AtCESA9* seed-specific RNAi lines ranged from 8.62%- 8.98%. The mean C16:0 content in *AtCESA1* seed-specific lines was 8.69% (lowest 8.5%; highest 8.85%) even though it was not a statistically significant change (P value, 0.06). C18:0 content, however, was not changed in any of *AtCESA* RNAi lines (*AtCESA1* seed-specific, *AtCESA9* seed-specific, *AtCESA9* constitutive). Furthermore, the proportion of C18:3 $\Delta^{9cis,12cis,15cis}$ (hereafter C18:3) in the seed oil of *AtCESA9* constitutive RNAi lines increased up to 16.43% (lowest 16.37%; highest 16.73%; P value 0.01) from 16.05% (lowest 15.7%; highest 16.74%) in the control seeds, where the change in C18:3 content was significant (Table 4.1). A similar trend was observed for C20:0,

C20:1Δ^{11cis} (hereafter C20:1), C20:2$\Delta^{11cis,14cis}$ (hereafter C20:2), C20:3$\Delta^{11cis,14,cis,14cis}$ (hereafter C20:3) and C22:1Δ^{13cis}(hereafter C22:1) content in *AtCESA9* constitutive T_3 seeds. In contrast, the C18:1Δ^{9cis} (hereafter C18:1)and C18:1Δ^{11cis} content of the oil of in *AtCESA9* constitutive T_3 seeds displayed a slight reduction with P value of 0.01 in the student's T-test when compared to empty vector control.

Generally, the proportions of C16:0 or C16:1 in the oil of *AtCESA1*seed-specific and *AtCESA9* seed-specific RNAi T_3 seeds were slightly increased compared to control seeds. Furthermore, there were slight increases in long chain FAS (C16 and C18) and VLCFAs (> C18) in *AtCESA9* constitutively down-regulated seeds.

Collectively, there were slight changes in the FA compositions in *AtCESA* RNAi lines compared to control lines. In particular, there was a common pattern of FA compositional changes in seed-specific *AtCESA* down-regulated lines (*AtCESA1*ss and *AtCESA9*ss). The FA compositions of the seed oil of *AtCESA9* constitutive RNAi lines were somewhat different from the seed-specific down-regulated lines.

Table 4.1 Average fatty acid compositions with standard deviation (±SD) of the seed oil of *AtCESA1* and *AtCESA9* RNAi lines (T_3 seeds)

Data are mean with ±SD of seed samples of 7-14 independent lines with RNAi cassettes driven by the ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and driven by constitutive tCUP promoter for *AtCESA9* gene (CES9c). Fatty acid composition is presented as a percentage of each fatty acid of the total fatty acids on a mol% basis. Data were analyzed by one-tailed student's T-test. (▲/▼) indicates the values greater/lower than empty vector control at α=0.025 level.

Fatty acid	**Fatty acid composition (mol %)**			
	Con	CESA1ss	CESA9ss	CESA9c
C16:0	8.6 (±0.14)	8.69 (±0.13)	8.85 (±0.1) ▲	8.64 (±0.08)
C16:1	0.36 (±0.02)	0.39 (±0.02) ▲	0.39 (±0.04)	0.36 (±0.01)
C18:0	4.18 (±0.07)	4.14 (±0.1)	4.17 (±0.08)	4.17 (±0.06)
C18:1	16.37 (±0.6)	16.46 (±0.76)	16.31 (±0.55) ▼	15.83 (±0.37) ▼
C18:1c11	1.65 (±0.07)	1.61 (±0.03)	1.67 (±0.03)	1.59 (±0.05) ▼
C18:2	24.46 (±0.35)	24.3 (±0.3)	24.6 (±0.12)	24.3 (±0.2)
C18:3	16.05 (±0.29)	16 (±0.3)	15.97 (±0.39)	16.43 (±0.19) ▲
C20:0	2.43 (±0.05)	2.43 (±0.06)	2.43 (±0.06)	2.49 (±0.06)
C20:1	18.79 (±0.31)	18.86 (±0.22)	18.47 (±0.18) ▼	18.96 (±0.14)
C20:2	2.52 (±0.07)	2.52 (±0.09)	2.54 (±0.06)	2.6 (±0.07)
C20:3	0.42 (±0.01)	0.42 (±0.02)	0.42 (±0.02)	0.44 (±0.02) ▲
C22:1	1.93 (±0.06)	1.98 (±0.06)	1.93 (±0.05)	2.01 (±0.06) ▲

4.4 Down-regulation of *AtCESA1* in developing Arabidopsis seeds had a minor effect on seed protein content

To determine the effect of *AtCESA* down-regulation on seed protein content, selected T_3 homozygous seeds were analyzed for protein content using a modified "Dumas" method in the LECO nitrogen analyzer. As shown in Figure 4.7A, only the seeds with seed-specific down-regulation of the *AtCESA1* gene exhibited a slight 3% relative increase (P value; 0.02) in protein content (w/w) compared to control seeds. On average, the protein content in *AtCESA1* seed-specific RNAi lines was 28.4% (lowest 27.46%; highest 29.31% while most of the samples ranged from 28.4% - 29.3%) compared to 27.6% (lowest 25.80%; highest 29.68%, while most of the samples ranged from 26.1% - 28.4%) found in control lines. Neither constitutive nor seed-specific down-regulation of *AtCESA9* significantly affected the protein content of T_3 Arabidopsis seeds. On average, the seed protein content of *AtCESA9* seed-specific or constitutive RNAi lines was 27.45% (lowest 24.47%; highest 29.79%) or 27.71% (lowest 26.09%; highest 28.20%), respectively, compared to 27.6% found in control lines.

The seed protein content analysis of homozygous plant lines coming from same T_2 seed lines with insertion at one locus in the Arabidopsis genome indicated that all three *AtCESA1* seed-specific RNAi isogenic lines displayed minor enhanced protein content compared to control lines. CESA1ss4, CESA1ss18 and CESA1ss15 seeds exhibited 3.5%, 3% and 2% relative increases in protein content, respectively (Figure 4.7B). There was no observable effect of constitutive *ATCESA9* down-regulation on seed protein content in any of tested lines although seed- specific down- regulation resulted in slight variability in protein content among parental lines

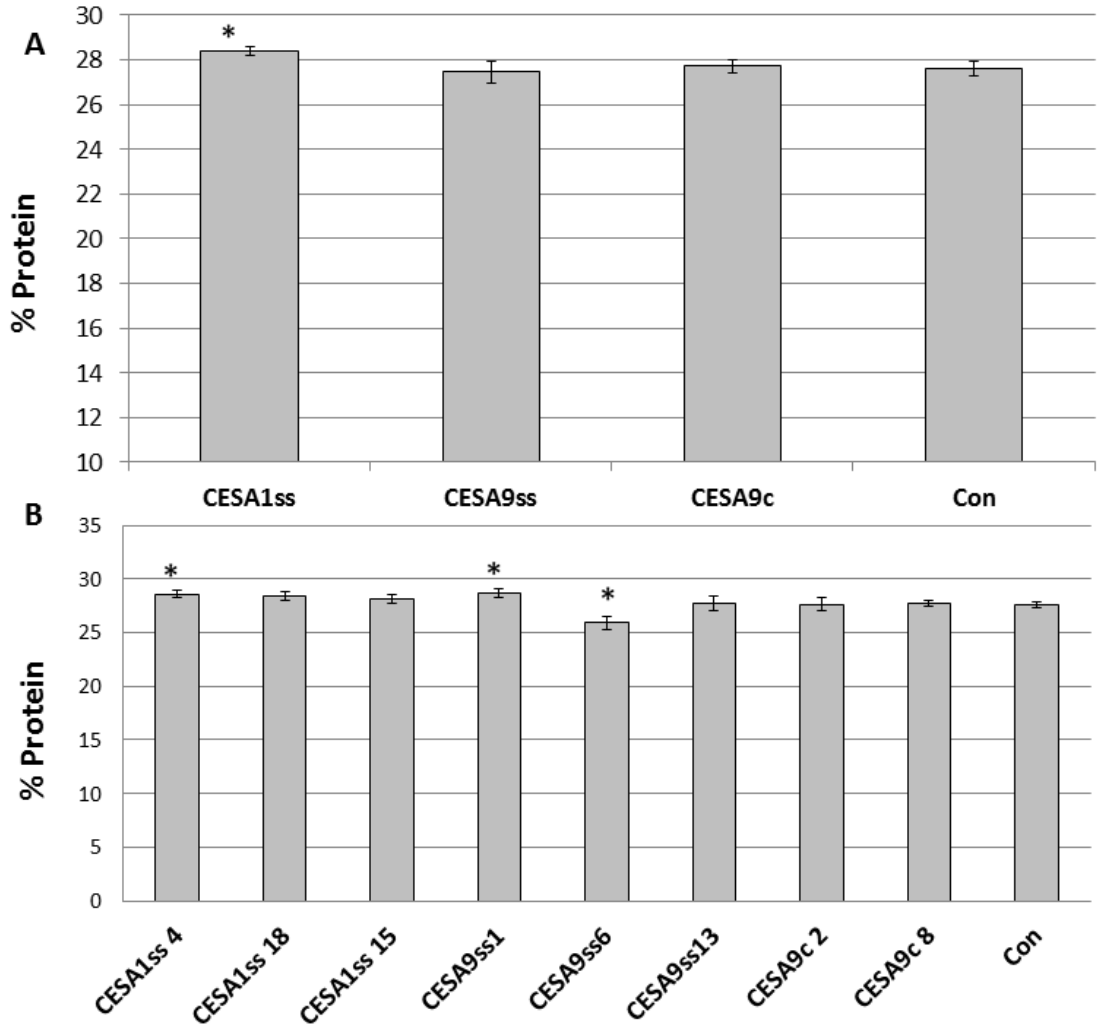

Figure 4.7 Total protein content as percentage of dry seed weight from T_3 homozygous Arabidopsis seeds transformed with RNAi cassettes driven by ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and driven by constitutive tCUP promoter for *AtCESA9* gene (CESA9c). (A) Each bar represents the average of 7-14 transgenic T_3 seed samples coming from plant lines transformed with corresponding construct. (B) Each bar represents the average of 3-4 transgenic T_3 seed samples coming from plant lines which have single copy DNA insertion in the same locus. In both graphs, error bars represents the standard error of the mean. Controls (Con) are the plants transformed with empty vector (In Both A and B represent the average of 6-11 biological repeats). A significant difference in seed protein content compared to the control is marked with *[$P \leq 0.05$; in one-tailed student's T-test]

4.5 *AtCESA* down-regulation resulted in reduced crystalline cellulose content of T_3 seeds

Even though the down-regulation of *AtCESA* genes showed minor effects on seed oil content, protein content or seed weight in the current study, the reductions in transcript levels of *AtCESA1* or *AtCESA9* genes in developing T_2 and T_3 seeds suggested that there should be reduced cellulose biosynthesis in the seeds. Previous studies have reported reduced cellulose contents in Arabidopsis seeds by down-regulating *AtCESA1* or *AtCESA9* (Beeckman et al., 2002; Stork et al., 2010) . In the current study, cellulose content was determined using a modified version of the Updegraff (1969) method. Hemicellulose was first removed from the samples using Updergraff reagent and the remaining crystalline cellulose was converted to glucose. Glucose was determined using the anthrone colorimetric assay. The relative amounts of acid-insoluble Glc (relative estimate of crystalline cellulose) were examined in seeds of RNAi and control lines. Acid-insoluble glucose levels are a quantitative estimate of crystalline cellulose since the glucose is derived from strong acid hydrolysis of crystalline cellulose. The average of total acid-insoluble glucose content in seeds of all three down-regulated lines were less than those of the empty vector controls, and highest reduction was observed for *AtCESA9* constitutive RNAi lines (P value; 6.78×10^{-5}) (Figure 4.8A). On average, the level of crystalline cellulose was reduced by ~25% or ~17% for *AtCESA1* or *AtCESA9* seed-specific RNAi lines respectively compared to the empty vector control line. Acid-insoluble glucose content, which represents the cellulose content, however, fluctuated greatly among different RNAi lines which were tested within groups and between groups (*CESA1* seed-specific, lowest 32.08 µg/ mg: highest 58.55 µg/ mg, P value 0.03; *CESA9* seed-specific, lowest 40.14 µg/ mg: highest 66.76 µg/ mg, P value 0.03; *CESA9* constitutive, lowest 35.33 µg/ mg: highest 41.95 µg/ mg).

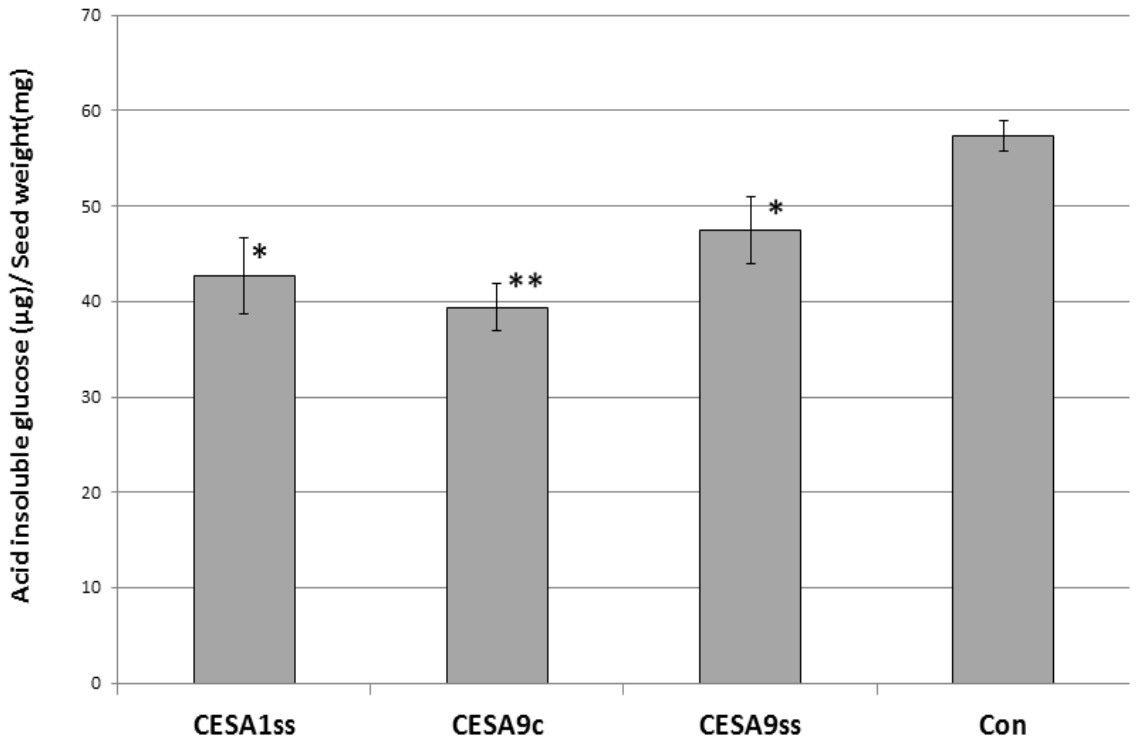

Figure 4.8 Acid insoluble glucose content of T_3 homozygous Arabidopsis seeds transformed with RNAi cassettes driven by ß-phaseolin promoter for: *AtCESA1* (CESA1ss), *AtCESA9* gene (CESA9ss) and under the control of constitutive tCUP promoter for *AtCESA9* gene (CESA9c). Each bar represents the average of 4-6 transgenic T_3 seed samples coming from plant lines transformed with the corresponding construct. The error bars represent the standard errors of the means. Controls (Con) are the plants transformed with empty vector. A significant difference in acid insoluble glucose content in transgenic seeds compared to the control is marked with *($P \leq 0.05$) and very significant with **($P \leq 0.01$) in one-tailed student's t-test.

5. Discussion

Increasing the seed oil and/or protein content of oil crops is paramount importance to the seed oil industry (Weselake et al., 2009). Over the last several years, numerous studies have addressed this aspect. Diverting carbon flow from other metabolic pathways to seed oil and protein biosynthetic pathways has been explored to some extent as means of increasing seed oil and/or protein content (discussed in section 2.6).

Cellulose is a major component of the plant cell wall which depends on a substantial amount of carbon for its biosynthesis (Haigler et al., 2001). As mentioned previously, the cellulosic cell wall reduces the economic and nutritional value of oilseed meal due to comparatively higher levels of fibre that limits its inclusion in animal rations (Canola meal feed industry guide, 2009). A few recent studies have reported on the development of canola lines with less dietary fibre, with more protein and sucrose, through conventional breeding (Jia et al., 2012; Wickramasuriya et al., 2015).

Research described in this thesis focused on examining the effect of down-regulation of two different cellulose synthase genes (*AtCESA1* and *AtCESA9*) on the seed oil, protein and cellulose content of Arabidopsis seed. This research project tested the hypothesis that down-regulation of the production of cellulose within seed tissues might redirect carbon flow to oil and/or protein biosynthesis from cellulose biosynthesis. In turn, the resulting meal would be expected to have less fibre due to the decrease in cellulose content. Down-regulation of target genes was carried using an RNAi interference approach.

The results suggested that down-regulation of *AtCESA1* and *AtCESA9* led to an apparent reduction in the cellulose content of Arabidopsis seeds, whereas *AtCESA9* constitutive down-

regulation resulted in the greatest reduction in cellulose content (Figure 4.8). None of the down-regulation experiments resulted in increased seed oil content, but *AtCESA1* seed-specific down regulated lines resulted in a slight apparent increase in protein content (Figure 4.5). The current discussion will include possible strategies for utilizing *AtCESA* down-regulation as a molecular engineering tool to yield high quality oilseeds with higher oil and protein content accompanied by reduced complex fibre content.

As mentioned in the literature review, a number of previous studies indicated that *AtCESA1* or *AtCESA9* mutants produce reduced amounts of cellulose in either primary or secondary cell wall in Arabidopsis. Many of these reports stated that available cellulose biosynthesis mutants have severe defects in plant morphogenesis with characteristic symptoms such as tissue swelling, cell wall gaps, altered vascular morphology and aberrant cell wall thickenings with ectopic deposition of callose and lignin (Arioli et al., 1998; Gillmor et al., 2002; Schrick et al., 2004; Somerville, 2006; Caño-Delgado et al., 2003). These characteristics affect plant growth in several ways including abnormal root growth, shorter internode length, small size leaves, abnormal vascular tissue swellings and dwarf phenotypes in plants (Arioli et al., 1998; Gillmor et al., 2002; Caño-Delgado et al., 2003; Burton et al., 2000). Given the issues associated with cellulose biosynthesis mutants, seed-specific *AtCESA* RNAi constructs were generated and introduced to wild type plants in order to obtain *AtCESA1* and *AtCESA9* partial down-regulated transgenic lines. It was anticipated that a partial reduction of cellulose content might be achieved without impacting plant growth. *AtCESA9* constitutive RNAi lines were also generated because of a previous report which indicated the seed coat preferential expression of *AtCESA9* and its involvement in secondary cell wall cellulose biosynthesis in seed coat (Stork et al., 2010).

AtCESA1 gene down-regulation was confirmed with gene expression analysis in both T_2 and T_3 seeds. As expected, most of RNAi lines which have been tested resulted in somewhat reduced cellulose content in their seeds compared to empty vector control. In the current study, it appears that AtCESA1 seed-specific down-regulation did not have an effect on the vegetative growth of the plants (Figure 4.6). Absence of common cellulose deficient phenotypes in these lines could be due to seed-specific partial down-regulation of *AtCESA1* gene. Beeckman et al. (2002) reported that a very strong *AtCESA1* Arabidopsis mutant (*rsw1-2*, which seems to completely abolish the enzymatic activity) resulted in abnormal radial expansion in embryo. Furthermore, the investigators did not observe any change in overall cell arrangement in the hypocotyl and radicle, including the cell numbers in the apical-basal dimension in these seeds. In addition, they observed some abnormalities in vegetative tissues such as limited root growth and no trichomes in leaves. Furthermore, there were no apparent growth retardations in *AtCESA9* constitutively down-regulated plants (Figure 4.6). Similarly, Stork et al. (2010) observed that the *AtCESA9* T-DNA mutant did not affect either seedling growth or leaf size in Arabidopsis. Nonetheless, Stork et al. (2010) reported the presence of altered seed coat cell size, cell shape, and internal angle uniformity in *AtCESA9* mutants of Arabidopsis using scanning electron microscopy. The investigators suggested that these defects arose due to reduced cellulose content in the seed coats. In current study, only *AtCESA9* seed-specific T_3 RNAi seeds exhibited a minor reduction in seed weight (Figure 4.7). The minor reduction in seed weight may have been attributable to reduced overall deposition of cellulose and/or oil and protein. The reductions in cellulose content varied among T_3 homozygous lines. Previous studies on RNAi down-regulation of different genes in *Brassica* spp. indicated that different degrees of down-regulation were possible in the same population (Jagtap et al., 2011; Mietkiewska et al., 2008). This could be the

reason for the observed variations in cellulose content in Arabidopsis seeds of *AtCESA* RNAi line in the current study. Reduced cellulose contents were observed in both *AtCESA9* seed-specific and constitutive RNAi lines, whereas the highest reduction was observed in *AtCESA9* constitutive RNAi lines (Figure 4.8). The variability in *AtCESA9* gene down-regulation could be explained by T-DNA insertion at more than two loci of redundant DNA, as has been observed in CESA9ss-8 and CESA9c-1 lines in T_2 generation. Such T-DNA insertion at multiple loci could have affected the expression of *AtCESA9* RNAi cassettes. Furthermore, quite variable gene expression levels in those lines supported the fact that the effect of RNAi-mediated down-regulation can be quite variable within the same population (Jagtap et al., 2011; Mietkiewska et al., 2008).

Some studies have suggested that transcript level does not always reflect the level of protein produced by the cell. For example, Wijekoon and Facchini (2012) observed some variations in the correlation between transcripts and metabolite levels in virus-induced gene silencing of morphine pathway enzymes in *Papaver somniferum*. As suggested by Beeckman et al. (2002), the *AtCESA9* gene may have a unique temporal expression pattern in the Arabidopsis embryo. The investigators indicated that *AtCESA9* gene expression gradually decreased as the embryo matured and reached undetectable levels based on a DIG-labeled RNA *in-situ* hybridization assay conducted on the bent-cotyledon stage. This unique expression pattern of the *AtCESA9* gene may also cause for the observed expression variability in RNAi lines.

As mentioned previously in the literature review, the biosynthesis of seed storage compounds and cell wall components, such as cellulose, depends on the supply of carbon from the maternal plant tissues. The biochemical interactions between these pathways, however, are not fully understood. Sucrose which enters into the cell is hydrolyzed by the catalytic action of

either SUS or INV. The resulting hexoses can either enter glycolysis to produce pyruvate or can be utilized by CSC in the plasma membrane to synthesize cell wall components including cellulose (Baud et al., 2008; Haigler et al., 2001). When the cellulose biosynthesis pathway is attenuated, the biosynthesis of storage reserves, such as oil and/or protein, might be affected.

Increased seed oil content was not observed in any of the transgenic lines (*AtCESA1* seed-specific RNAi, *AtCESA9* seed-specific RNAi, *AtCESA9* constitutive RNAi) studied (Figure 4.3 and 4.4). Instead, there was an apparent reduction in seed oil content in *AtCESA1* seed-specific RNAi seeds (Figure 4.3 and 4.4) compared to empty vector control. Even though this was a slight reduction (~1%) compared to empty vector control, this observation was consistent across the generations studied. Down-regulated *AtCESA9* seed-specific and constitutive T_2 and T_3 seeds also showed some reduction in seed oil content amounting to less than 1% absolute reduction. It is interesting to note that the vast majority of studies using transgenic oilseed lines to increase seed oil content have only resulted in small increases (Singer et al., 2013; Liu et al., 2016). For example, overexpression of a cDNA encoding GPAT9, which catalyzes the first reaction in the Kennedy pathway leading to TAG, resulted in only a 1% absolute enhancement in seed oil content (Singer et al., 2016). Furthermore, the investigators did not observe a significant change in the seed oil of *GPAT9* RNAi seeds compared empty vector control lines. In another study, which targeted homomeric Arabidopsis ACCase to the plastids of developing *B. napus* seeds resulted in a small, but heritable, 5 % relative increase in seed oil content (Roesler et al., 1997).

Schrick et al. (2012) have observed a close relationship between cellulose biosynthesis and the sterol composition of the plasma membrane. Sterols are one of major lipid classes in the plasma membrane (Grosjean et al., 2015). Schrick et at. (2012) have noted that three Arabidopsis

sterol biosynthesis mutants namely *fackel* (*fk*), *cephalopod/sterol methyl transferase 1* (*cph/smt1*) and *hydra1* (*hyd1*) exhibited phenotypic defects which are typical for a cellulose deficiency. Furthermore, they have proposed that sterol composition and its availability in the plasma membrane may be critical for maintaining the correct subcellular localization, structural integrity and/or activity of the cellulose synthase machinery and cellulose accumulation in Arabidopsis (Schrick et al., 2004, 2012). Additionally, a previous study has shown that sitosterol-β-glucosides, act as primers for the attachment of glucose monomers during the synthesis of β−(1→4) glucan chains that form the cellulose microfibrils *in vitro* using crude membranes from cotton fibres (Peng et al., 2002). Specifically, radioactive sterol cellodextrins were synthesized by incubation of sitosterol-[^{14}C]glucoside (SG) and non-radioactive UDP-glucose (Peng et al., 2002). Others, however, have argued that SG is not important in cellulose synthesis since there was no correlation between sitosterol levels and cellulose content in the study of Schrick et al. (2004), and it was shown that the sitosterol-β-glucosides mutants did not affect cellulose biosynthesis in Arabidopsis plants (DeBolt et al., 2009; Kumar and Turner, 2015). Recently, Schrick et al. (2012) have emphasized the importance of investigating the interaction of sterols with the cellulose synthase machinery, either direct or indirect. Even though several studies have shown that lipid or FA-synthesising mutants show a negative effect on cell wall synthesis, there is no evidence available for cellulose synthesizing mutants, which show an effect in seed oil content (Schrick et al., 2012, 2004; Li et al., 2015). Several studies have specified the highly regulated manner of sterol biosynthesis through feedback mechanisms in plants as well as in animal cell lines. These studies suggest that the sterol levels rise when there is no available sink and the resulting sterols may bind to sterol biosynthetic enzymes and down-regulate their activity (Harker et al., 2003; DeBose-Boyd, 2008; Grosjean et al., 2015). Thus, sterol reduction could

affect the total lipid content of the seed. It may also be worthwhile to investigate sterol content and its composition in total oil of *AtCESA* RNAi lines.

Somewhat increased protein content was observed in *AtCESA1* seed-specifically down-regulated T_3 seeds (Figure 4.5.). This may due to the redirection of carbon-flux into protein biosynthesis rather than FA biosynthesis, especially if there is a feedback effect on FA synthesis and total lipid accumulation. Furthermore, a negative correlation between protein and fibre content has been demonstrated in black and yellow-seeded canola-type *B. napus* (Slominski et al., 2004; http://www.agwest.sk.ca/kaizen/CIM-AGCI/CIM2010/SlominskiCIM2010LowFibreCanola.pdf). An increase in carbon flux for protein biosynthesis, however, would also depend on available nitrogen (Baud et al., 2008).

Carbon flux effects in oilseeds could also be considered in terms of other cell wall materials (Stork et al., 2010) and the interplay of their formation with cellulose biosynthesis (Burton et al., 2000). Several previous studies have shown that when cellulose biosynthesis is inhibited in either the primary cell wall or secondary cell wall, the process is compensated by other cell wall polymers, such as hemicellulose and pectin. This happened because of the plant's attempt to overcome structural weakness in cell walls, which arose due to reduced cellulose content in Arabidopsis *AtCESA9* and *AtCESA1* mutants (Stork et al., 2010; Somerville, 2006; Burton et al., 2000). Stork et al. (2010) have observed an increase in neutral sugars in the total of cell wall material in *AtCESA9* mutant Arabidopsis seeds compared to control lines. Furthermore, Burton et al. (2000) observed an increase in homogalacturonan content of the cell walls of *Nicotiana benthamiana* leaves, which were compromised in cellulose content using virus-induced gene silencing of *CESA* genes (Burton et al., 2000). Thus, these studies suggest there are feedback loops interconnecting the cellular machinery controlling cellulose, pectin and

hemicellulose biosynthesis (Burton et al., 2000; Stork et al., 2010). In addition, Stork et al. (2010) reported a proportional increase in cell wall polymers other than cellulose, such as aliphatic monomers and some polyesters, in the seeds of *AtCESA9* mutants. In particular, the investigators observed an increase in C16:0 content and some other long chain FA, 18:1 dicarboxylic acid (DCA), 18:2 DCA, 18:1 and 18:2 ω-hydroxy Fas, along with two ester-bound aromatics (ferulate and sinapate). In the current study, some minor changes in FA composition were observed in *ATCESA* RNAi lines similar to what was found by Stork et al. (2010) (Table 4.1). Slight elevations were observed in C16:0 and C18:1 content in *AtCESA1* seed-specific RNAi seeds, and in C16:0 content in *AtCESA9* seed-specific RNAi lines. The degree of variations in FA compositions was more abundant in *AtCESA9* constitutive RNAi seeds (Table 4.1). There were slightly reduced amounts of C18:1, the 11*cis* isomer of C18:1 and enhanced C18:3, C20:0, C20:1, C20:2, C20:3, and C22:1 observed in *AtCESA9* constitutive RNAi seeds. It has been suggested that long chain and VLCFA species are much more abundant in the Arabidopsis seed coat and endosperm than in the embryo (Molina et al., 2006). The *AtCESA9* constitutive RNAi construct used in this study was expected to have more effect on cellulose biosynthesis in the seed coat when compared to seed-specific constructs designed using the embryo specific phaseolin promoter and terminator. As previously suggested by Stork et al. (2010), cellulose deficiency in the seed coat due to the down-regulation of *AtCESA9* may result in altered polyester monomer compositions. Further studies will be needed to confirm these changes since the differences in FA composition observed in the current study were minor.

It is important to note that most of the seed coat lipid polyester species (cutin and suberin) are composed of esterified hydroxy and polyhydroxy FAs in Arabidopsis and *B. napus* seeds (Molina et al., 2006). The level of these polyesters, however, is very low when compared

to the overall content of TAG in the seed (Molina et al., 2006). It may be worthwhile examining the effect of down-regulation of *AtCESA* genes on the lipid polyester content of the seed coat.

A complete analysis of the cell wall components of *AtCESA* RNAi seeds would be useful to understand how the cellulose defect affects other cell wall components. Since obtaining seeds with reduced complex fibre content is one of the objectives of this project, it would be necessary to find out whether the cellulose reduction actually reduces the complex fibre components in the cell walls. In previous studies with Arabidopsis seedlings, it has been suggested that reduced cellulose content may be compensated through increased production of other cell wall materials such as lignin (Schrick et al., 2012; Caño-Delgado et al., 2003).

By reducing cellulose biosynthesis in this study, it was expected that other metabolic pathways would draw upon the available carbon for use in storage lipid and/or protein biosynthesis. In the current study, however, there was a minor reduction in seed oil content in *AtCESA* RNAi lines and thus partial down-regulation of cellulose biosynthesis may be not sufficient to enhance total oil production unless combined with other approaches. The hexose-phosphates generated through sucrose hydrolysis need to be metabolized through the glycolytic pathway before the carbon can be utilized for FA biosynthesis (Baud et al., 2008). Since FA are synthesized within the plastid, Glc-6-P and PEP generated through the cytosolic glycolytic pathway need to be imported into the plastid before subsequent conversion to pyruvate and then to FA (Baud et al., 2008). Thus, it may be possible to enhance FA biosynthesis by over-expressing genes involved in cytoplasmic glycolysis and transport of Glc-6-P and PEP into the plastid (Meyer et al., 2012). In a previous study, down-regulation of starch synthesis on its own did not lead to increased oil content in Arabidopsis leaf tissues, unless accompanied by expression of cDNA encoding theWRINKLED1 (WRI1) transcription factor, which up-regulates

genes encoding enzymes in glycolysis and FA biosynthesis (Sanjaya et al., 2011). Overexpression of genes encoding glycolytic enzymes, Glc-6-P or PEP transporters or WRI1 combined with *CESA* down-regulation might result in significant increases in seed oil content. Even further increases in seed oil content might occur if genes encoding Kennedy pathway enzymes, such as GPAT or DGAT, are also co-expressed. Vanhercke et al. (2014) achieved high levels of leaf TAG through a combination of genetic engineering interventions which included up-regulation of FA biosynthesis, increased production of DGAT1 and reduced turnover of TAG. Introducing oilbody protein into the leaf resulted in the apparent protection of oilbodies from attack by TAG lipase. The strategy was referred to as 'push', 'pull' and 'protect'. Thus, combining *AtCESA* down-regulation with modification of push, pull and protect processes might lead to greater increases in seed oil than can be achieved with only push, pull and protect. The new approach could be referred to as divert, push, pull and protect. Furthermore, a recent study has demonstrated increased seed oil content could be obtained through down-regulation of a gene encoding TAG lipase, which minimized the lipid turnover (van Erp et al., 2014). The investigators showed that seed-specific overexpression *WRI1* and *DGAT1* combined with suppression of the TAG lipase *SUGAR-DEPENDENT1* resulted in a higher percentage of seed oil content and greater seed mass than manipulation of each gene individually.

Increasing the FA content in the cell could lead to a decrease in *de novo* fatty acid synthesis due to feedback mechanisms (reviewed by Ohlrogge and Jaworski, 2003). Intracellular accumulation of plastidial C18:1 has been shown to result in feedback inhibition of *de novo* FA biosynthesis in microspore-derived cell suspension cultures of *B. napus* (Andre et al., 2012). The investigators introduced a variety of Tween esters (polyethylene sorbitol esters) in the growth medium and observed maximum inhibition of *de novo* FA biosynthesis with feeding of Tween

esters containing C18:1 followed by Tween ester containing C16:0. Thus, it may be useful to combine *CESA* down-regulation with a desensitization of plastidial FA biosynthesis to increased C18:1 levels as a possible means of boosting seed oil content.

Most of the oxaloacetate produced from PEP in glycolysis is committed to the synthesis of Asp and amino acids derived from it (Baud et al., 2008). Since there was a trend for increasing protein content in *AtCESA1* RNAi seeds compared to empty vector control seeds, one might also expect to increase seed protein content by increasing the efficiency of glycolysis (Meyer et al., 2012). Thus, multi-gene interventions could also useful in enhancing storage protein content in oil seeds. Since protein biosynthesis mainly depends on nitrogen flux to the maturing embryo from the maternal plant, increasing nitrogen supply with *AtCESA* down-regulation would be a logical way to obtain higher protein content in oil seeds. A combination of *AtCESA1* down-regulation with overexpression of genes that improve the nitrogen utilization efficiency (NUE) of crop plants could also be an effective strategy to obtain oil seeds with higher protein content (McAllister et al., 2012). McAllister et al. (2012) suggested that the candidate NUE genes could be involved in different pathways relating to N uptake, assimilation, amino acid biosynthesis, C/N storage and metabolism, signalling and regulation of N metabolism and translocation, remobilization and senescence. For example, overexpression of two aminotransferase enzymes, asparagine amino- transferase (AspAT) and alanine amino-transferase (AlaAT), in Arabidopsis resulted in increased seed amino acid and protein content in seeds (Murooka et al., 2002; McAllister et al., 2012).

Thus, generally increasing available carbon for FA synthesis and AA synthesis by down-regulating metabolic pathways that compete for available carbon may not be sufficient to increase the storage lipid or protein content (Vanhercke et al., 2014; Shewry et al., 1995). Down-

regulation of *AtCESA1* and *AtCESA9* genes may only be an effective strategy for decreasing fibre content and increasing oil and/or protein content by combining down-regulation with other metabolic interventions such as those described above. The *AtCESA1* seed-specific RNAi lines could be particularly useful since these lines showed a slight tendency for increased protein and reduced cellulose content. The *AtCESA9* constitutive RNAi lines could also be possible targets due to preferential effects in the seed coat combined with no effect on plant growth (Stork et al., 2010). In combining *CESA* down-regulation with other metabolic interventions, it would be critical to demonstrate that these collective interventions do not seriously comprise plant growth.

6. Conclusions and Future Directions

In this project, three RNAi constructs (RNAi cassettes driven by seed-specific ß-phaseolin promoter for: *AtCESA*1, *AtCESA9* genes and driven by constitutive tCUP promoter for *AtCESA9* gene) were developed and introduced into Arabidopsis wild type plants to determine if reduced cellulose deposition could result in increases in seed oil and/or protein content. In addition, these transgenic lines might be expected to have decreased fibre content. Down-regulation of *AtCESA1* and *AtCESA9* led to a reduction in the cellulose content of Arabidopsis seeds. These results suggest that down-regulation of cellulose synthesis in oil crops represents a potentially useful strategy for increasing the digestibility of the meal obtained during extraction of the seed oil. Despite the possibility of extra carbon being available from reduced cellulose deposition in Arabidopsis, there was no increase in seed oil content in any of the transgenic lines. There was, however, a tendency towards reduced seed oil content for the *AtCESA1* and *AtCESA9* seed-specific RNAi lines. In addition, seed-specific down-regulation of *AtCESA1* resulted in a slight increase in seed protein content.

In future studies, it would be useful to analyze and quantify other components of the cell wall in the above transgenic lines to investigate whether reduced cellulose deposition results in the reallocation of carbon to other complex carbohydrates such as hemicellulose. It would also be interesting to evaluate the ultra-structure of the cell wall using microscopy techniques.

In addition, transcriptomic and metabolomic analysis of developing seeds of Arabibopsis could prove useful in gaining insights into the relationship between cellulose, storage lipid and protein biosynthesis in control seeds versus transgenic seeds, wherein cellulose biosynthesis has been partially down-regulated. This exercise could potentially provide clues regarding additional

gene targets to implement in a multi-gene approach to increasing seed oil and/or protein content. Recently, extensive research has been conducted on the implementation of multi-gene strategies to boost oil content in seeds and vegetative tissue. Thus, a number of apparently logical gene targets are already available. For example, it may be useful to enhance glycolysis, fatty acid production, TAG assembly and reduce TAG turnover in combination with down-regulation of cellulose synthase in the seed coat and/or embryo. This approach could develop a more effective 'sink' for carbon diverted from deposition into cellulose. In terms of increasing protein content, it may be useful to increase NUE in combination with down-regulation of cellulose synthesis.

References

Alexander, D. and Seif, R. (1963). Relation of kernel oil content to some agronomic traits in maize. Crop Sci.: 354–355.

Andre, C., Haslam, R.P., and Shanklin, J. (2012). Feedback regulation of plastidic acetyl-CoA carboxylase by 18:1-acyl carrier protein in Brassica napus. Proc. Natl. Acad. Sci. **109**: 10107–10112.

Andriotis, V.M.E., Pike, M.J., Kular, B., Rawsthorne, S., and Smith, A.M. (2010). Starch turnover in developing oilseed embryos. New Phytol. **187**: 791–804.

Arioli, T., Peng, L., Betzner, A.S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R.E. (1998) Molecular Analysis of Cellulose Biosynthesis in Arabidopsis. Science. **279**: 717–720

Barratt, D.H.P., Derbyshire, P., Findlay, K., Pike, M., Wellner, N., Lunn, J., Feil, R., Simpson, C., Maule, A.J., and Smith, A.M. (2009). Normal growth of Arabidopsis requires cytosolic invertase but not sucrose synthase. Proc. Natl. Acad. Sci. U. S. A. **106**: 13124–13129.

Bates, P.D. and Browse, J. (2011). The pathway of triacylglycerol synthesis through phosphatidylcholine in Arabidopsis produces a bottleneck for the accumulation of unusual fatty acids in transgenic seeds. Plant J. **68**: 387–399.

Baud, S., Dubreucq, B., Miquel, M., Rochat, C., and Lepiniec, L. (2008). Storage Reserve Accumulation in Arabidopsis: Metabolic and Developmental Control of Seed Filling. Arab. B. **6**: doi: 10.1199/e0113.

Beeckman, T., Przemeck, G., Stamatiou, G., Lau, R., Terryn, N., Rycke, R., Inze, D., and Berleth, T. (2002). Genetic complexity of cellulose synthase A gene function in Arabidopsis embryogenesis. Plant Physiol. **130**: 1883–1893.

Brown JR, R. (1996). The biosynthesis of cellulose. J.M.S. Pure Appl. Chem. **A33**: 1345–1373.

Burn, J.E., Hocart, C.H., Birch, R.J., Cork, A.C., and Williamson, R.E. (2002). Functional Analysis of the Cellulose Synthase Genes. Plant Physiol. **129**: 797–807.

Burton, R.A., Gibeaut, D.M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D.C., and Fincher, G.B. (2000). Virus-induced silencing of a plant cellulose synthase gene. Plant Cell **12**: 691–706.

Caño-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in Arabidopsis thaliana. Plant J. **34**: 351–362.

Canola meal feed industry guide (2009). canola council. www.canolacouncil.org. **4th Edition**.

Carroll, A., Mansoori, N., Li, S., Lei, L., Vernhettes, S., Visser, R.G.F., Somerville, C., Gu, Y., and Trindade, L.M. (2012). Complexes with mixed primary and secondary cellulose synthases are functional in Arabidopsis plants. Plant Physiol. **160**: 726–37.

Chapman, K.D. and Ohlrogge, J.B. (2012). Compartmentation of triacylglycerol accumulation in plants. J. Biol. Chem. **287**: 2288–2294.

Chen, G., Woodfield, H.K., Pan, X., Harwood, J.L., and Weselake, R.J. (2015). Acyl-Trafficking during Plant Oil Accumulation. Lipids **50**: 1057–1068.

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

Cosgrove, D.J. (2005). Growth of the plant cell wall. Nat. Rev. Mol. Cell Biol. **6**: 850–861.

DeBolt, S. et al. (2009). Mutations in UDP-Glucose:sterol glucosyltransferase in Arabidopsis cause transparent testa phenotype and suberization defect in seeds. Plant Physiol. **151**: 78–87.

DeBose-Boyd, R.A. (2008). Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. Cell Res. **18**: 609–21.

Desprez, T., Juraniec, M., Crowell, E.F., Jouy, H., Pochylova, Z., Parcy, F., Höfte, H., Gonneau, M., and Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A. **104**: 15572–7.

Dyer, J.M., Stymne, S., Green, A.G., and Carlsson, A.S. (2008). High-value oils from plants. Plant J. **54**: 640–655.

Ekman, A., Hayden, D.M., Dehesh, K., Bülow, L., and Stymne, S. (2008). Carbon partitioning between oil and carbohydrates in developing oat (Avena sativa L.) seeds. J. Exp. Bot. **59**: 4247–4257.

Endler, A. and Persson, S. (2011). Cellulose synthases and synthesis in arabidopsis. Mol. Plant **4**: 199–211.

Van Erp, H., Kelly, A.A., Menard, G., and Eastmond, P.J. (2014). Multigene engineering of triacylglycerol metabolism boosts seed oil content in Arabidopsis. Plant Physiol. **165**: 30–6.

Fick, G.N. (1983). Genetics and breeding of sunflower. J. Am. Oil Chem. Soc. **60**: 1252–1253.

Foster, C.E., Martin, T.M., and Pauly, M. (2010). Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part I: Lignin. J. Vis. Exp.: doi: 10.3791/1837.

Frisch, D.A., Geest, A.H.M. Van Der, Dias, K., and Hall, T.C. (1995). Chromosomal integration is required for spatial regulation of expression from the phaseolin promoter. Plant J. **7**: 503–512.

Fujiwara, T., Nambara, E., Yamagishi, K., Goto, D.B., and Naito, S. (2002). Storage Proteins. Arab. B. **25**: doi: 10.1199/e0020.

Gillmor, C.., Poindexter, P., Lorieau, J., Palcic, M.M., and Somerville, C. (2002). α-glucosidase I is required for cellulose biosynthesis and morphogenesis in Arabidopsis. J. Cell Biol. **156**: 1003–1013.

Goderis, I.J.W.M., De Bolle, M.F.C., François, I.E.J.A., Wouters, P.F.J., Broekaert, W.F., and Cammue, B.P.A. (2002). A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. Plant Mol. Biol. **50**: 17–27.

Griffiths, J.S., Tsai, A.Y.-L., Xue, H., At Alin Voiniciuc, C., Šola, K., Seifert, G.J., Mansfield, S.D., and Haughn, G.W. (2014). SALT-OVERLY SENSITIVE5 Mediates Arabidopsis Seed Coat Mucilage Adherence and Organization through Pectins. Plant Physiol. **165**: 991–1004.

Grosjean, K., Mongrand, S., Beney, L., Simon-Plas, F., and Gerbeau-Pissot, P. (2015). Differential effect of plant lipids on membrane organization specificities of phytosphingolipids and phytosterols. J. Biol. Chem. **290**: 5810–5825.

Haigler, C.H., Ivanova-Datcheva, M., Hogan, P.S., Salnikov, V. V, Hwang, S., Martin, K., and Delmer, D.P. (2001). Carbon partitioning to cellulose synthesis. Plant Mol. Biol. **47**: 29–51.

Harker, M., Hellyer, A., Clayton, J.C., Duvoix, A., Lanot, A., and Safford, R. (2003). Co-ordinate regulation of sterol biosynthesis enzyme activity during accumulation of sterols in developing rape and tobacco seed. Planta **216**: 707–715.

Herman, E. and Larkins, B. (1999). Protein storage bodies and vacuoles. Plant Cell **11**: 601–614.

Higgins, T.J. V (1984). Synthesis and regulation of major protein in seed. Ann. Rev. Plant Physiol. **35**: 191–221.

Iyer, V. V., Sriram, G., Fulton, D.B., Zhou, R., Westgate, M.E., and Shanks, J. V. (2008). Metabolic flux maps comparing the effect of temperature on protein and oil biosynthesis in developing soybean cotyledons. Plant, Cell Environ. **31**: 506–517.

Jagtap, U.B., Gurav, R.G., and Bapat, V.A. (2011). Role of RNA interference in plant improvement. Naturwissenschaften **98**: 473–492.

Jia, W., Mikulski, D., Rogiewicz, A., Zdunczyk, Z., Jankowski, J., and Slominski, B.A. (2012). Low-fiber Canola. Part II: Nutritive Value of the Meal. J. Agric. Food Chem. **60**: 12231-12237.

Jiang, Y. and Deyholos, M.K. (2010). Transcriptome analysis of secondary-wall-enriched seed coat tissues of canola (Brassica napus L.). Plant Cell Rep. **29**: 327–342.

Keegstra, K. (2010). Plant Cell Walls. Plant Physiol **154**: 483–486.

Kennedy, C.J., Cameron, G.J., Sturcova, A., Apperley, D.C., Altaner, C., Wess, T.J., and Jarvis, M.C. (2007). Microfibril diameter in celery collenchyma cellulose: X-ray scattering and NMR evidence. Cellulose **14**: 235–246.

Kim, M.J., Yang, S.W., Mao, H.-Z., Veena, S.P., Yin, J.-L., and Chua, N.-H. (2014). Gene silencing of Sugar-dependent 1 (JcSDP1), encoding a patatin-domain triacylglycerol lipase, enhances seed oil accumulation in Jatropha curcas. Biotechnol. Biofuels **7**: 36.

Kimura, S., Laosinchai, W., Itoh, T., Cui, X., Linder, C.R., and Brown, R.M. (1999). Immunogold Labeling of Rosette Terminal Cellulose-Synthesizing Complexes in the Vascular Plant Vigna angularis. Plant Cell **11**: 2075–2085.

Kumar, M. and Turner, S. (2015). Plant cellulose synthesis: CESA proteins crossing kingdoms. Phytochemistry **112**: 91–99.

Li, N., Gugel, I.L., Giavalisco, P., Zeisler, V., Schreiber, L., Soll, J., and Philippar, K. (2015). FAX1, a Novel Membrane Protein Mediating Plastid Fatty Acid Export. PLoS Biol. **13**: 1–37.

Lin, Y., Ulanov, A. V., Lozovaya, V., Widholm, J., Zhang, G., Guo, J., and Goodman, H.M. (2006). Genetic and transgenic perturbations of carbon reserve production in Arabidopsis seeds reveal metabolic interactions of biochemical pathways. Planta **225**: 153–164.

Liu, Q. et al. (2016). Genetic enhancement of oil content in potato tuber (Solanum tuberosum L.) through an integrated metabolic engineering strategy. Plant Biotechnol. J.: 1–12.

Marillia, E.F., Micallef, B.J., Micallef, M., Weninger, A., Pedersen, K.K., Zou, J., and Taylor, D.C. (2003). Biochemical and physiological studies of Arabidopsis thaliana

transgenic lines with repressed expression of the mitochondrial pyruvate dehydrogenase kinase. J. Exp. Bot. **54**: 259–270.

Mcallister, C.H., Beatty, P.H., and Good, A.G. (2012). Engineering nitrogen use efficient crop plants: The current status. Plant Biotechnol. J. **10**: 1011–1025.

Meyer, K., Stecca, K.L., Ewell-Hicks, K., Allen, S.M., and Everard, J.D. (2012). Oil and Protein Accumulation in Developing Seeds Is Influenced by the Expression of a Cytosolic Pyrophosphatase in Arabidopsis. Plant Physiol. **159**: 1221–1234.

Mietkiewska, E., Hoffman, T.L., Brost, J.M., Giblin, E.M., Barton, D.L., Francis, T., Zhang, Y., and Taylor, D.C. (2008). Hairpin-RNA mediated silencing of endogenous FAD2 gene combined with heterologous expression of Crambe abyssinica FAE gene causes an increase in the level of erucic acid in transgenic Brassica carinata seeds. Mol. Breed. **22**: 619–627.

Molina, I., Bonaventure, G., Ohlrogge, J., and Pollard, M. (2006). The lipid polyester composition of Arabidopsis thaliana and Brassica napus seeds. Phytochemistry **67**: 2597–2610.

Murasnige, T. and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Agsays with Tohaoco Tissue Cultures. Physiol. Plant **15**: 473–497.

Murooka, Y., Mori, Y., and Hayashi, M. (2002). Variation of the amino acid content of Arabidopsis seeds by expressing soybean aspartate aminotransferase gene. J. Biosci. Bioeng. **94**: 225–230.

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**: 285–293.

NSW Department of Primary Industries (2014). Variability of quality traits in canola seed , oil and meal - a review.: 1–26.

Ohlrogge, J.B. and Jaworski, J.G. (2003). Regulation of fatty acid synthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 109–138.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., and Browse, J. (1994). Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell **6**: 147–158.

Otegui, M.S., Herder, R., Schulze, J., Jung, R., and Staehelin, L.A. (2006). The proteolytic processing of seed storage proteins in Arabidopsis embryo cells starts in the multivesicular bodies. Plant Cell **18**: 2567–2581.

Peng, L., Kawagoe, Y., Hogan, P., and Delmer, D. (2002). Siotsterol-beta-glucosidase as Primer for cellulose Sythesis in Plants. Science **295**: 147–150.

Persson, S., Paredez, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M., and Somerville, C.R. (2007). Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **104**: 15566–71.

Richmond, T.A. and Somerville, C.R. (2000). The cellulose synthase superfamily. Plant Physiol. **124**: 495–498.

Roesler, K., Shintani, D., Savage, L., Boddupalli, S., and Ohlrogge, J. (1997). Targeting of the Arabidopsis homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. Plant Physiol. **113**: 75–81.

Sanjaya, Durrett, T.P., Weise, S.E., and Benning, C. (2011). Increasing the energy density of vegetative tissues by diverting carbon from starch to oil biosynthesis in transgenic Arabidopsis. Plant Biotechnol. J. **9**: 874–883.

Schmittgen, T.D. and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. **3**: 1101–1108.

Schrick, K., Debolt, S., and Bulone, V. (2012). Deciphering the molecular functions of sterols in cellulose biosynthesis. Front. Plant Sci. **3**: 84.

Schrick, K., Fujioka, S., Takatsuto, S., Stierhof, Y.D., Stransky, H., Yoshida, S., and Jurrgens, G. (2004). A link between sterol biosynthesis, the cell wall, and cellulose in Arabidopsis. Plant J. **38**: 227–243.

Schwender, J. and Ohlrogge, J.B. (2002). Probing in vivo metabolism by stable isotope labeling of storage lipids and proteins in developing *Brassica napus* embryos. Plant Physiol. **130**: 347–361.

Schwender, J., Ohlrogge, J.B., and Shachar-Hill, Y. (2003). A flux model of glycolysis and the oxidative pentosephosphate pathway in developing Brassica napus embryos. J. Biol. Chem. **278**: 29442–29453.

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

Shewry, P.R., Napier, J. A, and Tatham, A. S. (1995). Seed storage proteins: structures and biosynthesis. Plant Cell **7**: 945–956.

Shi, L., Katavic, V., Yu, Y., Kunst, L., and Haughn, G. (2012). Arabidopsis glabra2 mutant seeds deficient in mucilage biosynthesis produce more oil. Plant J. **69**: 37–46.

Shockey, J., Regmi, A., Cotton, K., Adhikari, N., Browse, J., Bates, P.D., Wallace, J., and Buckler, E.S. (2015). Identification of Arabidopsis GPAT9 (At5g60620) as an Essential Gene Involved in triacyglycerol biosynthesis. Plant Physiol. **170**: pp.15.01563–.

Siloto, R.M.P., Findlay, K., Lopez-Villalobos, A., Yeung, E.C., Nykiforuk, C.L., and Moloney, M.M. (2006). The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. Plant Cell **18**: 1961–74.

Singer, S.D., Chen, G., Mietkiewska, E., Tomasi, P., Jayawardhane, K., Dyer, J.M., and Weselake, R.J. (2016). Arabidopsis GPAT9 contributes to synthesis of intracellular glycerolipids but not surface lipids. J. Exp. Bot.: doi:10.1093/jxb/erw242.

Slominski, B.A., Meng, X., Jia, W., Nyachoti, M., Jones, O., and Rakow, G. (2004). Chemical composition and nutritive value of yellow-seeded Brassica napus canola. Feed And Ind. Raw Mat. 253–255.

Somerville, C. (2006). Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol. **22**: 53–78.

Stadler, R., Lauterbach, C., and Sauer, N. (2005). Cell-to-Cell Movement of Green Fluorescent Protein Reveals Post-Phloem Transport in the Outer Integument and Identifies Symplastic Domains in Arabidopsis Seeds and Embryos. Plant Physiol. **139**: 701–712.

Stork, J., Harris, D., Griffiths, J., Williams, B., Beisson, F., Li-Beisson, Y., Mendu, V.,

Haughn, G., and Debolt, S. (2010). CELLULOSE SYNTHASE9 serves a nonredundant role in secondary cell wall synthesis in Arabidopsis epidermal testa cells. Plant Physiol. **153**: 580–9.

Tan, S.H., Mailer, R.J., Blanchard, C.L., and Agboola, S.O. (2011). Extraction and characterization of protein fractions from Australian canola meals. Food Res. Int. **44**: 1075–1082.

Taylor, N.G., Howells, R.M., Huttly, A.K., Vickers, K., and Turner, S.R. (2003). Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc. Natl. Acad. Sci. U. S. A. **100**: 1450–5.

Tomlinson, K.L., McHugh, S., Labbe, H., Grainger, J.L., James, L.E., Pomeroy, K.M., Mullin, J.W., Miller, S.S., Dennis, D.T., and Miki, B.L.A. (2004). Evidence that the hexose-to-sucrose ratio does not control the switch to storage product accumulation in oilseeds: Analysis of tobacco seed development and effects of overexpressing apoplastic invertase. J. Exp. Bot. **55**: 2291–2303.

Updegraff, D.M. (1969). Semimicro determination of cellulose in biological materials. Anal. Biochem. **32**: 420–424.

Wanasundara, J.P.D. (2011). Proteins of Brassicaceae oilseeds and their potential as a plant protein source. Crit. Rev. Food Sci. Nutr. **51**: 635–77.

Weber, H., Borisjuk, L., and Wobus, U. (1997). Sugar import and metabolism during seed development. Trends Plant Sci. **2**: 169–174.

Weichert, N. et al. (2010). Increasing sucrose uptake capacity of wheat grains stimulates storage

protein synthesis. Plant Physiol. **152**: 698–710.

Weigelt, K., Küster, H., Radchuk, R., Müller, M., Weichert, H., Fait, A., Fernie, A.R., Saalbach, I., and Weber, H. (2008). Increasing amino acid supply in pea embryos reveals specific interactions of N and C metabolism, and highlights the importance of mitochondrial metabolism. Plant J. **55**: 909–926.

Weselake, R.J., Taylor, D.C., Rahman, M.H., Shah, S., Laroche, A., McVetty, P.B.E., and Harwood, J.L. (2009). Increasing the flow of carbon into seed oil. Biotechnol. Adv. **27**: 866–878.

Wesley, S. V et al. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. **27**: 581–590.

Wickramasuriya, S.S., Yi, Y.-J., Yoo, J., Kang, N.K., and Heo, J.M. (2015). A review of canola meal as an alternative feed ingredient for ducks. J. Anim. Sci. Technol. **57**: 29.

Wijekoon, C.P. and Facchini, P.J. (2012). Systematic knockdown of morphine pathway enzymes in opium poppy using virus-induced gene silencing. Plant J. **69**: 1052–1063.

Yang, Q., Fan, C., Guo, Z., Qin, J., Wu, J., Li, Q., Fu, T., and Zhou, Y. (2012). Identification of FAD2 and FAD3 genes in Brassica napus genome and development of allele-specific markers for high oleic and low linolenic acid contents. Theor. Appl. Genet. **125**: 715–729.

Yoshie-Stark, Y., Wada, Y., and Wasche, A. (2008). Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. Food Chem. **107**: 32–39.

Appendix 1. Contribution to other projects

1. I was involved in a project in Dr. Weselake's lab aimed at characterizing type 9 Arabidopsis *sn*-glycerol-3-phosphate acyltransferase (GPAT9) which was operated in the Kennedy pathway leading to triacylglycerol accumulation. I developed some RNAi cassettes and some transgenic lines, and collected some morphological data. As result of my contribution, I became one of the co-authors of the following article: **Singer, S.D., Chen, G., Mietkiewska, E., Pernell, T., Jayawardhane, K., Dyer, J.M., Weselake, R.J. (2016). Arabidopsis GPAT9 contributes to synthesis of intracellular glycerolipids but not surface lipids. Journal of Experimental Botany, 67,4627–4638**

2. I also became involved in a Genome Canada Genomic Applications Partnership Project with Dow AgroSciences Canada Inc. The project title was "**Enhancement of Commercial Utilization of Canola Oil and Meal by Manipulation of Cellular and Sub-Cellular Metabolism Involving Fats and Carbohydrates**". My research on down-regulation of *AtCESA* genes was an integral part of this project. In addition to experimental work, I contributed to the preparation of progress reports and presentations at project meetings occurring on August 5, 2015, August 18, 2016 and October 3, 2016. The first two project meetings took place at Dow AgroSciences headquarters in Saskatoon with the most recent meeting occurring at our University.

3. The following conference abstracts resulted from my research on AtGPAT9:

    3.1 **Singer, S.D., Chen, G., Mietkiewska, E., Tomasi, P., Dyer, J.M., Jayawardhane, K., and <u>Weselake, R.J.</u>** (2015). A functional characterization

of *GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 9* (*GPAT9*) from the model oilseed plant, *Arabidopsis thaliana*. *Botany 2015*, Edmonton, Alberta, Canada; July 25-29 (oral presentation by SDS)

3.2 **Singer, S.D., Chen, G., Mietkiewska, E., Tomasi, P., Jayawardhane, K., Dyer, J.M., and Weselake, R.J.** (2015). GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (GPAT9) contributes to glycerolipid biosynthesis in various tissues of *Arabidopsis thaliana*. *11th International Symposium on Biocatalysis and Agricultural Biotechnology*, Banff, Alberta, Canada; September 13-16 (oral presentation by SDS)

3.3 **Jayawardhane, K.N., Singer, S.D., and Weselake, R.J.** (2015). Overview of known strategies to boost oil content in seeds. *Phytola Science Meeting*, Banff, Alberta, Canada; September 17 (poster presentation by KNJ)

3.4 **Weselake, R.J., Singer, S.D., Chen, G., Mietkiewska, E., Tomasi, P., Jayawardhane, K., and Dyer, J.** (2016). Arabidopsis GPAT9 catalyzes the acylation of the *sn*-1 position of glycerol-3-phosphate in the Kennedy pathway. *Northern Great Plains Lipids Conference*, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota, June 11-12 (oral presentation by RJW)

3.5 **Jayawardhane, K., Singer, S.D., Chen, G., Mietkiewska, E., Tomasi, P., Dyer, J., and Weselake, R.J.** (2016). Contribution of glycerol-3-phosphate acyltransferase (GPAT9) in glycerolipid biosynthesis in different plant tissues. *Plant Biotech 2016, Joint Meeting of the Canadian Society of Plant Biologists*

and the Canadian Association for Plant Biotechnology, Queen's University, Kingston, Ontario, June 19-21 (oral presentation by KJ)

3.6 **Singer, S., Chen, G., Mietkiewska, E., Tomas, P., Jayawardhane, K., Dyer, J., and <u>Weselake, R.J.</u>** (2016). The properties of Arabidopsis GPAT9 are consistent with the enzyme's operation in the Kennedy Pathway. *22nd International Symposium on Plant Lipids*, Göttingen, Germany, July 3-8 (oral presentation by RJW)