Lipids in Seeds of Oat (*Avena* spp.), a Potential Oil Crop

Content, Quality, Metabolism,
and Possibilities for Improvement

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Abstract

New oil crops with high yield and oil content are urgently needed. Oat is the only cereal that accumulates substantial amount of lipids in the endosperm. This gives it potential as an oil crop, which could address both nutritional and environmental concerns of modern society. To develop oat with increased oil content, thorough investigations on its lipid metabolism and tools for genetic manipulations are needed.

Analyses of lipids in seeds of wild and cultivated oat species revealed variation in wild oat accessions in both oil content and quality. This variation should allow for development of new oat varieties for diverse applications. However, to develop oat as an oil crop, a level of 20% of the seed dry weight as oil is required and the range of oil content in the studied accessions was 4-10%. Another feature which showed almost no deviation was the amount of omega 3 fatty acid, α-linolenic acid (0.6-2.1%). To develop oat varieties with ultra-high seed oil and increased content of omega 3 fatty acids, a biotechnological approach is preferred to conventional breeding methods.

Lipids in oat endosperm have been reported to exist, not enclosed in oil bodies, but as non-structured oil smear. In my study, I also observed oil smears, a phenomenon probably correlated with the reduced amount of oil body associated proteins in the endosperm, as revealed by microscopic and staining methods. This was supported experimentally by SDS-PAGE separation of oil-body proteins and immunoblotting and immunolocalization with antibodies against a 16 kD oil body protein.

Biochemical studies on lipid mobilization during oat seed germination demonstrated efficient utilization of oil reserves from the starchy endosperm; these results were corroborated by microscopy. An oat cultivar which is capable of accumulating high amounts of oil in the endosperm was shown to be also efficient in utilizing these reserves upon germination.

Agrobacterium-mediated transformation is one of the methods for modification of oat oil content and/or its quality. The results of the analyses aimed at development of an *Agrobacterium*-mediated oat transformation protocol are presented in this thesis.

Keywords: *Agrobacterium*-mediated transformation, *Avena*, cereals, fatty acid, germination, lipid, oat, oil crop, regeneration, tissue culture

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To my family

Allt blir bra till slut och blir det inte bra så är det inte slut än (Everything will be fine at the end and if it is not fine then it is not the end yet)

Susanne Persson

Contents

List of Publications

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

I Leonova S., Shelenga T., Hamberg M., Konarev A.V., Loskutov I., Carlsson S. A. (2008). Analysis of oil composition in cultivars and wild species of oat (*Avena* sp.). *Journal of Agricultural and Food Chemistry* 56, 7983-7991.

II Heneen K.W., Karlsson G., Brismar K., Gummesson P-O., Marttila S., Leonova S., Carlsson S.A., Bafor M., Banas A., Mattsson B., Debski H., Stymne S. (2008). Fusion of oil bodies in endosperm of oat grains. *Planta* 228, 589-599.

III Leonova S., Grimberg Å., Marttila S., Stymne S., Carlsson S.A. (2010). Mobilisation of lipid reserves during germination of oat (*Avena sativa* L.), a cereal rich in endosperm oil. *Journal of Experimental Botany* 61(11), 3089-3099.

IV Leonova S., Carlsson S.A., Zhu L-H. Optimization of regeneration and transformation protocols for oat (manuscript).

The contribution of Svetlana Leonova to the papers included in this thesis was as follows:

I Planned and performed the experiments and evaluated the data, wrote the paper together with co-authors

II Took part in the experimental work and in writing the paper

III Planned and performed a large part of experimental work and analyses of the data, wrote the paper together with co-authors

IV Planned and performed experimental work and evaluation of the data, wrote the manuscript together with co-authors

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ACP	acyl carrier protein
ATP	adenosine triphosphate
BAP	6-benzylaminopurine
cv	cultivar
CoA	co-enzyme A
DAG	diacylglycerol
dw	dry weight
ER	endoplasmic reticulum
FA	fatty acid
FAD	flavine adenine dinucleotide
FFA	free fatty acid
GUS	β-glucuronidase
GPAT	glycerol-3-phosphate acyltransferase
IME	immature embryo
IAA	indole-3-acetic acid
kDa	lilodalton
LM	light microscopy
NAA	naphthaleneacetic acid
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
PC	phosphatidylcholine
PL	phospholipids
PCR	polymerase chain reaction
TAG	triacylglycerol
TDZ	thidiazuron
TLC	thin layer chromatography
ZR	zeatine riboside

1 Introduction

By 2050, Earth's population will likely reach nine billion people (UN 2004). The increasing population in combination with the gradual decrease in fossil oil reserves requires alternative sources of energy. Since fossil oil predominantly originates from plant oil, plant lipids resemble fossil oil in their chemical structure and therefore have the potential to replace it in many different applications. However, the production base for plant oils is very narrow; only four oil crops are responsible for more than 80% of the current global production. Furthermore, yield increases in these crops cannot satisfy the increasing demands of plant oils for food, feed and energy purposes. Therefore, new oil crops with high yield and oil content are urgently needed. To achieve this goal, efficient plant breeding techniques and especially genetic engineering are urgently needed.

How is intensive agriculture going to address the problem of food supplies? Among the most evident tasks are increasing total harvested biomass, lowering production costs, and increasing crops' nutritional value. Regarding nutrition, accumulation of lipids instead of proteins and carbohydrates in the crop seed is preferential. The energy value of proteins and carbohydrates is 16-17kJ g^{-1} while the level of lipids is much higher, about 37kJ g^{-1}. High oil content is one of the characteristic features of oat grain which makes oat a potential oil crop (EPOBIO 2011). Increasing the lipid content of oat seeds to 30% would translate into three-fold increase in energy content, as compared with seeds of existing oat varieties with average oil content of about 5-8% (Leonova et al. 2008; Liu 2011). Oat is a crop with well-developed agronomy and relatively small requirements for fertilizers, herbicides, pesticides, and with drought tolerant and winter hardy cultivars. In addition, oat seeds' 5-8% lipid content is already high compared with other cereals; total oil amounts in seeds of such crops as wheat, barley, rye, and triticale is 1-3% (Price & Parsons 1975; Ragaee et al. 2006; Liu 2011). Certain wild and cultivated oat species show

even higher oil concentrations, containing up to 10-13% (Lehtinen & Kaukovirta-Norja 2011) and lines with 18% oil have been reported (Frey & Holland 1999).

Food made out of oat is generally considered healthy, or so called *functional food* (Singh et al. 2013). It is rich in beta-glucans, polysaccharides which have been shown to reduce the risk of cardiovascular disease (Queenan et al. 2007; Daou & Zhang 2012). Oat is one of the few cereals that are possible to consume for a majority of people that suffer from celiac disease (Garsed & Scott 2007; Pawlowska et al. 2012). Seeds of oat contain high levels of antioxidants (avenanthramides and vitamin E), which have a range of positive effects on human health (Meydani 2009). Despite the various health benefits of oat as a food, its production has been declining steadily during last half-a-century, giving way to soybean, maize, rapeseed and other crops (FAOSTAT 2011). Breeding oat cultivars with increased oil content and/or improved fatty acid composition of oil would raise the interest of growers and consumers in this crop and increase the share of energy-rich and healthy food on the market. Studies of oat lipid metabolism are crucial to achieve this goal.

The aim of this study was to investigate different aspects of oat lipid metabolism such as: oil content and its lipid classes, fatty acid composition of oat cultivars and various wild oat species, distribution of oil in the grain and particularly in the starchy endosperm, and mobilization of lipid reserves in germinating oat seeds. Efforts to develop *Agrobacterium*-mediated oat transformation and improve oat regeneration protocols were also made.

2 Background

2.1 Characteristics of genus *Avena*

2.1.1 Biology and agronomy

Oat is an annual cool season C3 true grass with five geographical regions of origin: North-Western and Western Europe, Northern Africa, Mediterranean region, Southern-Eastern Asia, and the rest of Europe and Asia (Rodionova et al. 1994; Kelly & Sakhanokho 2008). Such wide geographical distribution probably explains its tolerance to the different climate conditions: oat can grow in both temperate regions as well as in subtropical areas. The genus *Avena* includes between 7 and 70 wild and cultivated oat species according to different authors and includes species with diploid (2n=14), tetraploid (2n=28), and hexaploid (2n=42) chromosomal sets (Rodionova et al. 1994; Kelly & Sakhanokho 2008; Loskutov 2008).

The genus *Avena* is a member of tribe *Aveneae* (monocotyledonous family *Poaceae*) and is not directly related to other cereals belonging to such tribes as *Triticeae* (wheat, rye, and barley), *Oryseae* (rice), and *Paniceae* (switchgrass). This may explain its biochemical differences from other cereals, such as high oil content, presence of specific antioxidants (avenanthramides) and proteins (avenins) which differ in their structure and properties from proteins of other cereals (Valentine 2011).

Oat is relatively resistant to several pests and diseases that damage other cereal crops, such as the fungus that causes take-all disease (Liu et al. 2001) and root rot (Mert-Türk 2005). However, it is susceptible to smut, crown and stem rust, head blight, some bacterial and viral diseases (Mielniczuk et al. 2004; Tekauz et al. 2004; Valentine 2011). This necessitates treatment of oat seeds with fungicides or selection of resistant varieties for cultivation. Since there are currently no available herbicides for oat, a well thought-out crop

rotation is recommended to ensure weed control in oat fields (Anonimous 2009).

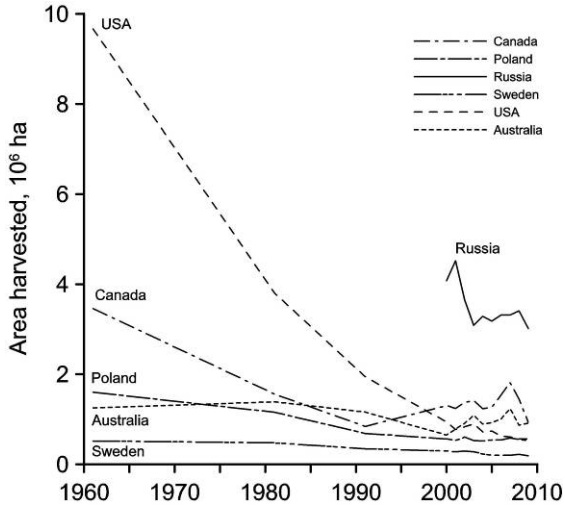

Figure 1. Chart on oat area harvested during last 50 years of several oat producers. Source: fao.org

Oat production (Fig. 1) is currently greatest in Russia followed by Canada, USA, Australia and several EU countries, mostly Finland, Sweden, and Poland (FAOSTAT 2011). The trend of declining acreage for oat production is obvious for all of the top oat producers except Australia where a tendency towards increased production has been observed over the period 2000-2009. The decline in oat production is directly linked to industrial development. Since workhorses were the main consumers of oats, its significance decreased in the era of machinery. However, promotion of oat as a very healthy product with many beneficial properties can play an important role in returning this traditional crop to our fields and markets.

2.1.2 Oat seed: structure and chemistry

Oat seed (also grain, kernel) in strict botanical view is a fruit, which in members of the *Poaceae* is called *caryopsis*, or groat. To be able to germinate and produce a new plant, the seed possesses several structures different in their functions and therefore in their morphology, physiology, and chemistry. The main constituents of the oat seed are bran, embryo, scutellum, aleurone layer and starchy endosperm. The kernel can be naked or covered by husks (hulls) which consist of outer lemma and inner palea.

The term *bran* used in milling industry designates outer layers of oat seeds under the hull: pericarp, seed coat (or testa), nucellus, aleurone layer, and part of the starchy endosperm adjacent to aleurone cells, called the subaleurone layer. The first three layers, i.e. pericarp, testa and nucellus, originate from the ovary and have no metabolic activity in the mature grain. They consist of

insoluble polysaccharides and autofluorescent compounds, probably phenolics (Miller & Fulcher 2011).

Seventy percent of the mature oat grain is made up of starchy endosperm (Youngs 1972). In cereals, this tissue serves as a site of nutrient accumulation and its cells undergo the programmed cell death at seed maturation (Young & Gallie 2000). Proteins, lipids, and carbohydrates, mainly starch and beta-glucans, stored in endosperm provide the growing embryo with energy and building material during early stages of germination when the photosynthetic apparatus is not yet established. Among oat endosperm reserves, lipid makes oat different from other cereals (Banas et al. 2007; Koehler & Wieser 2013). No other cereal crop accumulates oil in the endosperm in such amounts. Another distinguishing feature of oat endosperm lipids is their configuration as non-discrete fused structures rather than the discrete oil bodies present in living cells (Heneen et al. 2008). Starch is the main component of the oat grain, comprising 60% of its endosperm reserves (Zhou et al. 1999). In oat endosperm, starch content is negatively correlated with lipid content (Peterson & Wood 1997). Beta-glucan is a soluble fiber consisting of glucose moieties linked with each other by beta-glycosidic bonds. In oat seeds, beta-glucans account for 4-6% of seed dry weight (DW) (Lee et al. 1997; Sikora et al. 2013). In a mutagenized oat population, lines with beta-glucan content from 1.7 to 7.8% have been identified (Sikora et al. 2013). These polysaccharides are accumulated in the cell walls of the endosperm and, to a lesser extent, in the aleurone layer (Lazaridou & Biliaderis 2007; Sikora et al. 2013). Since no metabolic activity occurs in the endosperm cells of mature grain, endosperm reserves are broken down by enzymes secreted from surrounding cells of the embryo and aleurone layer over the course of germination.

Through most of its length, the aleurone layer is one cell thick. In contrast to endosperm cells, the aleurone layer is a living structure which supports metabolic activity in the mature grain (Evers et al. 1999). During seed germination, cells of the aleurone layer secret enzymes for hydrolysis of starchy endosperm reserves. The cell walls of the aleurone layer are very thick; they also contain beta-glucans, although in lesser amount than do starchy endosperm cells. The inner part of the aleurone cells contains structures called *aleurone grains*, i.e. proteins surrounded by lipids and organelles (ER, mitochondria, and plastids) (Bechtel & Pomeranz 1981; Peterson et al. 1985). Lipids in living cells of oat seed (i.e. cells of embryo, aleurone layer and scutellum) have the same oil body structure as lipids of other oil accumulating seeds. TAG is surrounded by a single layer phospholipid (PL) membrane which is penetrated by proteins, oleosins, caleosins, and steroleosin (Frandsen et al. 2001); oil bodies of oat also contain high levels of E-vitamins (White et

al. 2006). Cells of the aleurone layer accumulate phytin (salt of *myo*-inositol hexaphosphoric acid) and protein-carbohydrate bodies (Bechtel & Pomeranz 1981) consisting of complexes of carbohydrates, niacin, and aromatic amines (Fulcher et al. 1981). Phytin and niacin are among the health promoting features in oats.

The embryo is the smallest part of the oat seed. It consists of the embryonic axis and the scutellum, a modified second cotyledon of monocotyledonous plants. In the embryonic axis, two structures are distinguishable, coleoptile and coleorhiza, which are the precursors of leaves and roots, respectively.

Histologically, the scutellum is made of two types of cells, inner spherical parenchymal and outer elongated epithelial cells (Swift & Obrien 1972; Negbi 1984). The parenchyma serves mostly as nutrient storage. During germination, the vascular system is formed in this tissue for transportation of degraded storage compounds from the endosperm to the embryo. Scutellar epithelial cells then elongate and separate from each other to enlarge the contact area with endosperm compounds. These cells secrete hydrolyzing enzymes and absorb the released nutrients from the starchy endosperm (Swift & Obrien 1972; Negbi 1984; Negbi & Sargent 1986; Leonova et al. 2010).

2.2 Lipid metabolism

Lipids are a class of organic compounds exhibiting wide variation in chemical structure. It is therefore easier to define lipids on the basis of their physical properties as “compounds that are insoluble in water and that can be extracted from cells by nonpolar organic solvents (such as chloroform)” (Ohlrogge & Browse 1995). As a result of such a wide definition, lipids encompass such different chemical molecules as acylglycerols, saccharolipids, polyketides, sterols, and prenols. The first three are synthesized from ketoacyl subunit condensation (or in other words, FA-derived lipids) and the other two originate from isoprenoid biosynthetic pathway (Fahy et al. 2011). Due to the large number of molecules each lipid class comprises, I have chosen to focus my investigations on FA derived lipids, particularly triacylglycerols (TAG) as the main lipid class in oil accumulating seeds.

FA are 4-54 carbon long molecules with a carboxyl group in one end and a methyl group in the other end of highly reduced carbon chain. The numbering of carbon atoms in a FA molecule starts from the carboxyl group. FA can be saturated or unsaturated, i.e. possessing one or more double bonds between carbon atoms. A FA with more than one double bond is called polyunsaturated FA (PUFA). Two classes of PUFA are of particular nutritional significance for human beings. These are omega 3 FA where the first double bond occurs at

third carbon from the methyl group and omega 6 FA with the first double bond at sixth carbon.

Lipids in plants play various roles at all stages of development. Here, I focus mostly on TAG as important energy storage molecules in seeds of many plant species including oat. TAG is synthesized by esterification of a glycerol backbone with FA. The important intermediate in TAG formation is phospholipid (PL), a major component of plasma membrane. PL consists of a glycerol backbone esterified with FA in the sn-1 and sn-2 positions and with a phosphate group in the sn-3 position.

2.2.1 TAG biosynthesis

Lipid biosynthesis in plants can occur via the prokaryotic or the eukaryotic pathways. The prokaryotic pathway designates the lipid synthesis within plastids while the eukaryotic involves FA incorporation into different lipid classes in ER, FA editing, and transport of some lipids back to the plastid for further modifications.

De novo fatty acid biosynthesis

Fig. 2 summarizes FA synthesis and Fig. 3 shows FA assembly into different lipid classes. FA biosynthesis involves interaction of the following factors: acetyl-CoA as a carbon source, ATP (adenosine 5-three phosphate) as energy supplier, NADPH as a reducing agent, and two enzymatic systems to drive the reactions. There is no evidence for transport of acetyl-CoA into the plastid. Most probably plastid acetyl-CoA is produced specifically for FA synthesis with pyruvate as the main precursor (Johnston et al. 1997). The first enzymatic system, acetyl-CoA carboxylase (ACCase) (Konishi et al. 1996) carries out carboxylation of acetyl-CoA in two steps: the biotin moiety of BCCP (biotin carboxyl carrier protein) is carboxylated in an ATP- and bicarbonate-requiring reaction, and then this carboxylated biotin serves as a donor of carboxyl moiety for acetyl-CoA, converting the latter into malonyl-CoA (Harwood 1996). In grasses, ACCase exists in two isoforms (chloroplast and cytosolic) of a multifunctional protein, while in dicotyledonous plants this enzyme is present in chloroplasts as a multiprotein complex (Gunstone et al. 2005).

Further reactions of de novo FA synthesis are carried out by fatty acid synthase (FAS) (Harwood 1996), which in plants belongs to Type II FAS and exists as individual enzymes (transacylase, synthase, reductase, dehydratase) in a complex with acyl carrier protein (ACP) (Brown et al. 2006). Malonyl-CoA:ACP transacylase transfers malonyl moiety from CoA to ACP. The resulting malonyl-ACP and acetyl-CoA are the substrate for β-ketoacyl-ACP synthase, KAS III (it was discovered after KAS I and KAS II and therefore

received the number III). KAS III drives the production of acetoacetyl-ACP. Reduction of its *β*-carbonyl group to produce *β*-alcohol (at the expense of NADPH), dehydration, and a second reduction to saturate the double bond complete the formation of C4-ACP. Further condensation of C4-ACP with malonyl-ACP is carried out by KAS I and starts another round of reduction-dehydration-reduction reactions to form C6-ACP. KAS I drives elongation of the fatty acid chain to up to 16 carbons. The third type of KAS, KAS II, adds acetyl moiety from malonyl-CoA to C16-ACP thus producing C18-ACP (stearoyl-ACP) (Gunstone et al. 2007; Li-Beisson et al. 2010). In stearoyl-ACP a double bond can be introduced under action of Δ9 FA desaturase (FAD) forming oleoyl-ACP (Voelker & Kinney 2001; Nakamura & Nara 2004). Other modifications of FA (such as desaturation, elongation, acetylation, hydroxylation, and production of cycle-containing FA) can take place when an FA is esterified to a glycerol molecule. Such modification occurs either inside or outside the plastid on ER where enzymes for FA editing are situated. Plastidial Δ9 FAD is one of the few soluble enzymes among all the FA modifying enzymes. Thioesterases are enzymes which release formed FA of different chain length from ACP. Thioesterase A (FATA) is specific to 18:1-ACP whereas thioestarase B (FATB) preferentially utilizes saturated FAs. FAs either stay inside the plastid for lipid biosynthesis or are transferred to cytosol for further incorporation into TAG or PL with or without additional modifications.

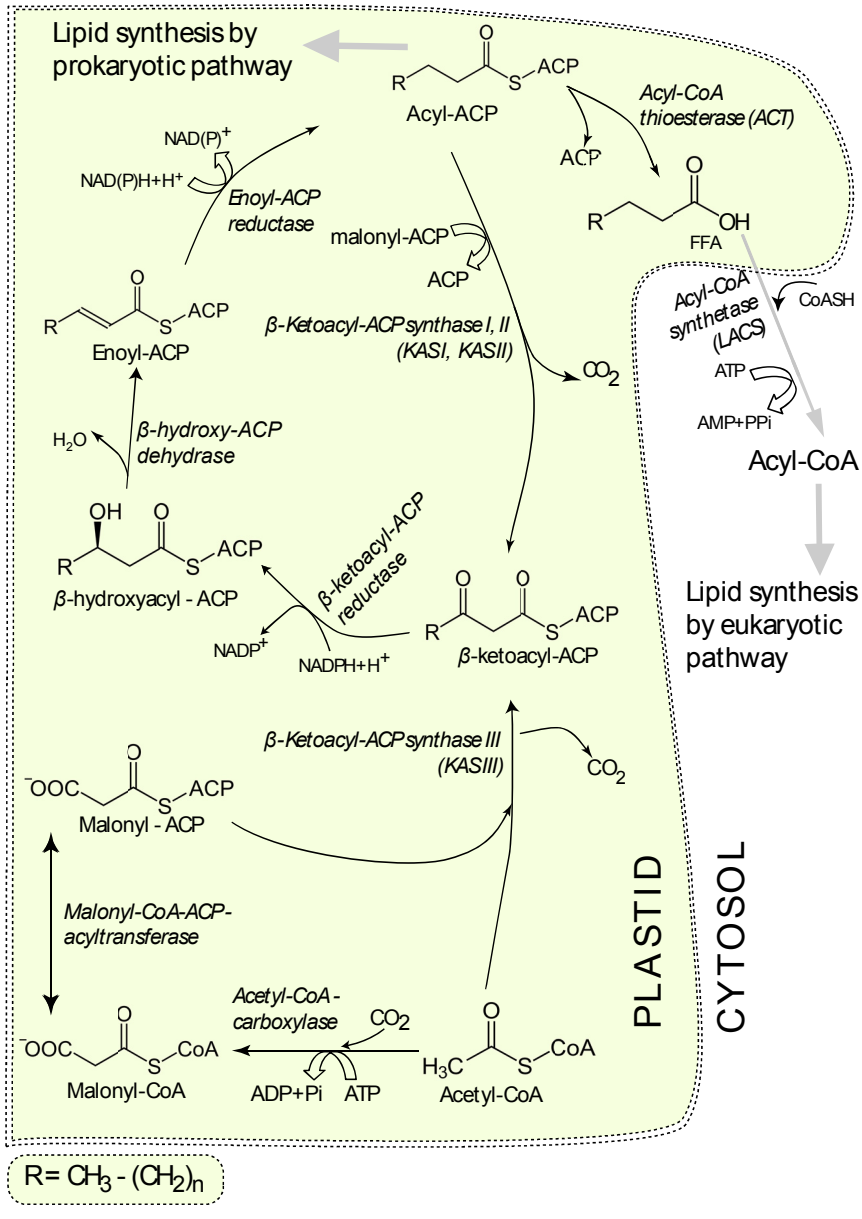

Figure 2. FA biosynthesis pathway in plants. The first condensation reaction is catalysed by *β*-ketoacyl-ACP synthase III (KAS III), which uses acetyl-CoA and malonyl-ACP substrates. The next six condensations are catalysed by KAS I and the final reaction between palmitoyl-ACP and malonyl-ACP is carried out by KAS II. Two carbon atoms are added to a FA chain in each cycle. Produced acyl-ACP can stay inside the plastid and be used for plastidial lipid synthesis or an acyl moiety can be freed from ACP and transported to cytosol where it is moved to ER as acyl-CoA for synthesis of TAG and PL.

Assembling of FA into different lipid classes

The mechanism of FA transfer through the plastid envelope remains unclear. Once outside the plastid, FA binds to CoA by long-chain-fatty-acid-acyl-CoA synthetase (LACS). The reaction requires ATP. Acyl-CoAs are substrate to ER localized enzymes producing complex lipids such as TAG and PL via the eukaryotic pathway. The precursor for both TAG and PL is diacylglycerol (DAG) which is synthesized from acyl-CoA via the Kennedy pathway. The initial step in this pathway is production of lysophosphatidic acid (LPA) by condensing an acyl moiety from acyl-CoA onto glycerol-3-phosphate (G3P) driven by glycerol-3-phosphate acyltransferase (GPAT). LPA is the substrate for LPA-acyl transferase (LPAAT), which utilizes another acyl-CoA to conjugate it onto the sn-2 position of LPA, producing phosphatidic acid (PA). Phosphatidate phosphohydrolase (PAP) removes phosphate from PA forming DAG. TAG is produced by three known pathways:

1) DAG is acylated onto the sn-3 position by diacylglycerol acyltransferase (DGAT) using acyl-CoA. With regards to its FA composition, TAG can differ markedly from membrane lipids (PL) of the same plant species demonstrating the selectivity of DGAT to certain FAs.
2) Formation of phosphatidylcholine (PC) by various routes (see below), e.g. by phosphatidylcholine: diacylglycerol acyltransferase (PDAT) (Dahlqvist et al. 2000). PC can be formed either via esterification of DAG in the *sn*-3 position with CDP-choline by choline phosphotransferase (CPT) or via acylation of glycero-phosphocholine (GPC) by GPC-acyltransferase (Stalberg et al. 2008). The formed lysoPC (LPC) is further acylated by LPCAT producing PC (Sperling & Heinz 1993). In both reactions, acylCoA is the acyl donor.
3) TAG from two DAG molecules is formed with release of sn-2 monoacylglycerol (MAG). For plants, this TAG formation was demonstrated in safflower (Stobart et al. 1997) but the enzyme, diacylglycerol:diacylglycerol transacylase, has only been purified from animal tissues (Lehner & Kuksis 1993).

Three isoforms of DGAT (DGAT 1, 2, and 3) have been described (Cases et al. 1998; Cases et al. 2001; Lardizabal et al. 2001; Saha et al. 2006; Shockey et al. 2006). All three isoforms have been identified in Arabidopsis as well as some other plants (Saha et al. 2006; Hernandez et al. 2012) The TAG-creating activity has been proven for DGAT1 (Li-Beisson et al. 2010) and DGAT3 (Hernandez et al. 2012). *dgat1* mutants showed a 40% decrease in the amount of oil in comparison with controls. Knockdown of *PDAT1* did not influence the amount of stored TAG (Katavic et al. 1995; Mhaske et al. 2005). The double

mutant *dgat1*/*pdat1* of Arabidopsis was not possible to obtain due to pollen lethality. Suppression of PDAT1 in *dgat1* background and suppression of DGAT1 in *pdat1* background via RNA interference resulted in 70-80% reduction of seed TAG (Zhang et al. 2009). Based on these results, it was proposed that DGAT1 is able to compensate lack of PDAT1 activity and *vice versa* (Li-Beisson et al. 2010). However, recent results showed that PDAT1 activity in *dgat1* background is strongly dependent on LPCAT2, which supplies PC inflow necessary for TAG biosynthesis (Xu et al. 2012). To further check whether LPCAT2 also plays a role in DGAT1 function the analyses of double mutant *pdat1/lpcat2* would be informative.

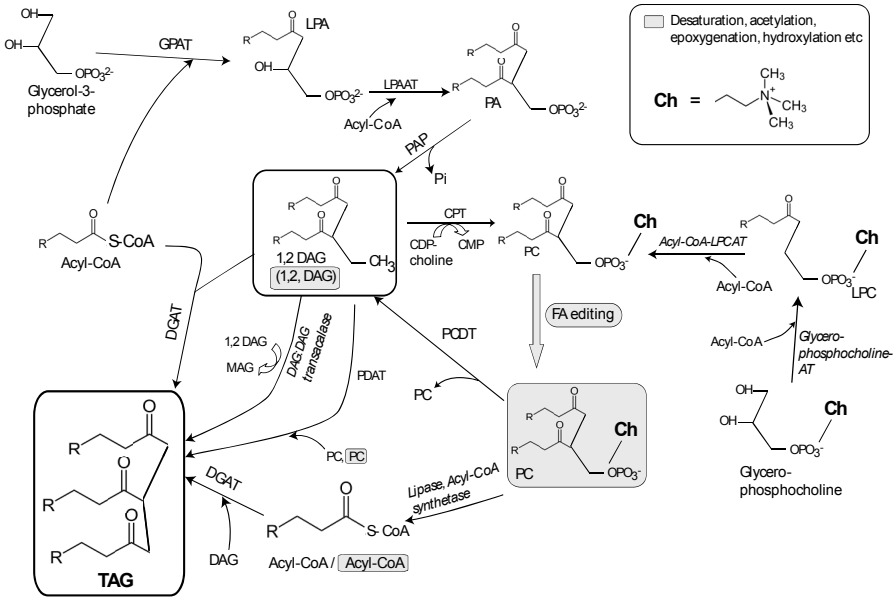

Figure 3. TAG synthesis in higher plants (takes place on the ER). Acyl-CoAs are esterified to glycerol-3-phosphate producing diacylglycerol (DAG) which is in turn can be converted either to phosphotidylcholine (PC) or to TAG via various routes. PC is an important compound since this is the site for diverse FA modifications. Abbreviations: GPAT – glycerol-3-phosphate acyltransferase; LPA – lysophospatidic acid; LPAAT – lysophosphatidic acid acyltransferase; PA - phosphatidic acid; PAP – Phosphatidate phosphohydrolase; CPT – choline phosphotransferase; CDP – cytidine diphosphate; CMP – cytidine monophosphate; PC – phosphatidylcholine; LPC – lysophosphatidylcholine; PCDT - phosphotidylcholine diacylglycerol transferase; PDAT - phosphatidylcholine: diacylglycerol acyltransferase; DGAT - diacylglycerol acyltransferase; LPCAT – lysophosphatidylcholine acyltransferase

TAG with modified FA

The enzymes for FA modifications are insoluble desaturase-like proteins located on the ER, with exception of a few soluble FADs in the plastid stroma

(Voelker & Kinney 2001). In seeds, modification of the FA moiety requires its presence on PC and in leaves - on monogalactosyldiacylglycerol (MGDG) produced by chloroplasts (Browse & Somerville 1991; Harwood 1996). PC and MGDG serve as substrates for various reactions of FA editing [e.g. introducing double and triple bonds, desaturation and acetylenation respectively, some cases of oxygenation (i.e. creating epoxy and hydroxy groups), and conjugation (Hildebrand 2010)]. The edited FAs are usually transported to TAG and not to PL probably due to deleterious effect of certain edited FA on the plasma membrane.

Several possible routes of FA transport from PC to TAG have been suggested (Fig. 3). Firstly, they can be relocated by the action of phosphotidylcholine diacylglycerol transferase (PCDT), which transfers a head group from PC to DAG (Lu et al. 2009). Such DAG with modified FA is then subject to DGAT or PDAT convertion into TAG. PCDT is encoded by *ROD1* (reduced oleate desaturation) and its knockout in Arabidopsis led to 41% reduction of 18:2/3 TAG in seeds.

Secondly, a modified acyl moiety from PC can be released by phospholipase (Athenstaedt & Daum 2006; Wang et al. 2012). Acyl-CoA synthetase forms acyl-CoA, which is then moved to TAG by DGAT. The remaining lysoPC is then subject to acyl-CoA:lysoPC acyltransferase, which produces PC.

Thirdly, modified FA from PC can be moved to TAG by PDAT, which transfers sn-2 acyl moiety from PC to DAG. This is a CoA-independent mechanism of TAG formation (Dahlqvist et al. 2000).

Substrate specificity of enzymes involved in TAG assembly and FA modification further complicates development of oil crops with novel FA.

2.2.2 Lipid degradation

Degradation of storage lipids

Release of energy stored in TAG involves two steps: cleavage of the FA from the glycerol backbone by action of lipases and catabolism of these FA via α-, β-, or ω-oxidation (Martin & Stumpf 1959; Mingrone & Castagneto 2006; Athenstaedt & Daum 2006; Hu et al. 2012; Theodoulou & Eastmond 2012; Lousa et al. 2013). Plant lipases have rather broad sequence structure with no clear indicators which would help in identification of lipolytic activity. Thorough studies with TAG as substrate are needed to confirm lipid hydrolytic capacity of 50-70 putative Arabidopsis TAG lipases, which have been discovered based on their sequence homology. Only four of TAG lipases have been characterized in detail (Padham et al.; He & Gan 2002; El-Kouhen et al.

2005; Ghosh et al. 2009; Seo et al. 2009). In particular, some TAG lipases have been shown to be active towards DAG and MAG as well as towards polar lipids and other substrates.

Regarding oat, high lipase activity, even in quiescent seeds, has been reported in a number of studies (Hutchinson et al. 1951; Sahasrabudhe 1982; Ekstrand et al. 1992; 1993). Lipolytic activity has been shown to be remarkably high in both bran (Martin & Peers 1953; Hu et al. 2009) and the inner endosperm (Lehtinen et al. 2003) although participation of these lipases in endosperm TAG degradation remains uncertain.

Oat lipases and other lipid hydrolytic enzymes (lipoxygenase and lipoperoxidase) are important factors affecting storage properties of oat products and may also be used as potential catalysts in applications requiring production and modification of FFA (Lehtinen & Kaukovirta-Norja 2011). To prolongate the shelf life of oat products, inactivation of the lipases by steam treatment has been proposed. Such treatment appears to be much more efficient than dry heat treatment, even though the temperature is the same (Doehlert et al. 2010).

β-oxidation

In higher plants, reactions of β-oxidation (Fig. 4) are carried out mostly in peroxysomes and glyoxysomes, while mitochondria are involved in breaking down branched FA (Li-Beisson et al. 2010). In peroxysomes, hydrogen peroxide (H_2O_2) is produced by oxidation of flavins, and glyoxysomes host the reactions of the glyoxylate pathway.

After release from the glycerol backbone by lipases, FAs are transported to peroxysomes by an ABC transporter protein and undergo activation by condensation with thiol group of CoA by LACS. FA β-oxidation consists of four principal reactions which are consequently repeated until the complete degradation of the FA (Galliard 1980; Gerhardt 1992; Graham & Eastmond 2002). During the first reaction, a hydrogen atom from carbon β (C-3) is removed by acyl-CoA oxidase and used to reduce FAD with hydrogen peroxide as the side product. At the second step, the double bond formed between α (i.e. the carbon next to carboxyl group) and β carbons in obtained 2*trans*-enoyl-CoA is hydrated by enoyl-CoA hydratase and produced L-*β*-hydroxyacyl-CoA is oxidized at β carbon by 3-hydroxyacyl-CoA dehydrogenase with simultaneous reduction of NAD^+. In the third reaction, these hydrolyzing and dehydrogenizing activities as well as some other reactions related to β-oxidation of unsaturated FA are carried out by a multifunctional protein (Poirier et al. 2006; Graham 2008). In the fourth

reaction, the bond between α and β carbons is cleaved by 3-ketoacyl-CoA thiolase and the obtained FA chain is shortened by two carbons. It is then attached to CoA and the cycle is repeated until the final product, acetyl-CoA, is produced (in case of odd-number FA the final product of β-oxidation is 3-carbon propionyl-CoA). By the glyoxylate cycle, acetyl-CoA is then converted to succinate which is the substrate for the tricarboxylic acid cycle in mitochondria.

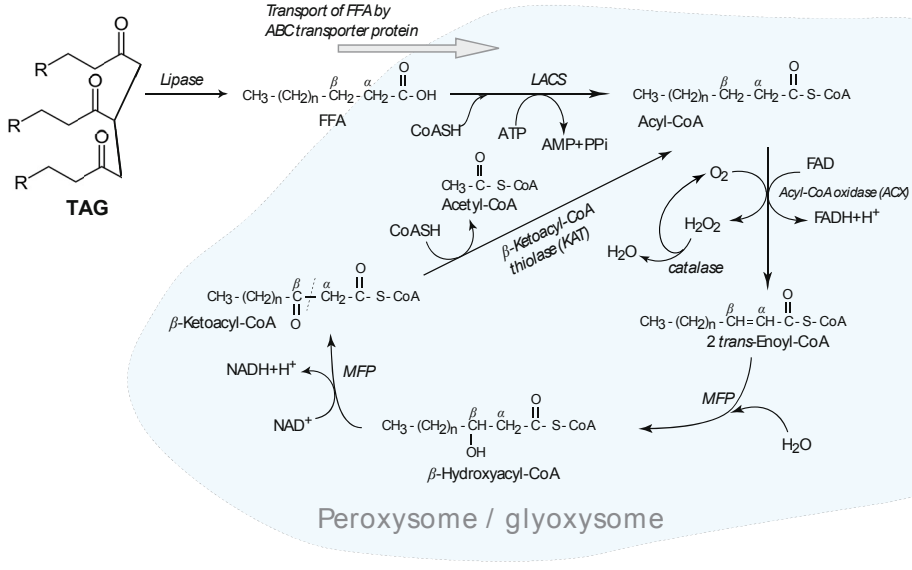

Figure 4. β-oxidation of FA in plants. FFA delivered to the peroxysome or glyoxysome by ABC transporter protein are bound to CoA by LACS and undergo reactions of dehydrogenation, hydration of the formed double bond, oxidation, and cleavage of the bond between α and β carbons. Remaining FA is shortened by two carbons and the cycle is repeated until degradation is complete. Multifunctional protein (MFP) consists of 2-*trans*-enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase.

In the case of unsaturated FAs, additional enzymes are needed to complete their degradation to acetate units. When the intermediate product of monounsaturated FA β-oxidation, cis-Δ3-dodecenoyl-CoA, is obtained the process cannot proceed. Presence of a double bond between C-3 and C-4 does not allow formation of a double bond between C-2 and C-3 required for preceding the β-oxidation. Isomerase that is able to convert cis-Δ3 double bond to trans-Δ2 double bond and hydratase that can utilize the obtained trans-enoyl:CoA for reduction are needed to overcome the problem. If FA contains more than one double bond, participation of another enzyme is required. When

the first double bond is cleaved as described above, trans-Δ2, cis -Δ4-dienoyl-CoA is produced. This is not an appropriate substrate for enoyl-CoA hydratase. Instead, 2,4-dienoyl-CoA reductase converts this product into, in the case of E.coli, trans-Δ2-enoyl-CoA (by the oxidation of NADPH), which is then subject to normal β-oxidation.

The rate of β-oxidation varies depending on the amount of substrate in the cell. Deficiency in DGAT activity or overexpression of thioesterase leads to accumulation of acyl-CoAs in the cytosol causing expression of enzymes involved in β-oxidation (Poirier et al. 1999).

β-oxidation in plants is a potential source of interesting intermediates. For example, polyhydroxyalkanoates (PHA) produced by polymerisation of hydroxyacyl-CoAs (by bacterial peroxysomal PHA synthases introduced into plants by genetic engineering) are environmentally friendly possible alternatives for plastic synthesis (Poirier et al. 1999).

Other types of FA breakdown reactions in plants include α- and ω-oxidation. The former, contrary to β-oxidation, takes place in both mitochondria and peroxysomes and is possibly involved in pathogen induced response. This suggestion is based on the observation that certain intermediates of α-oxidation are precursors of jasmonic acid as well as other important oxylipins (Hamberg et al. 1999). α-oxidation involves a single carbon atom being removed from the carbonyl end, forming an odd-number FA. Under certain conditions, 2-hydroxy FA are produced in this process (Gunstone et al. 2005). α-oxidation degrades substrates such as branched FAs carrying a methyl group at position 3 which prevents β-oxidation. After such FAs are modified via α-oxidation, they are subject to normal β-oxidation. ω-oxidation utilizes normal FA for production of dicarboxylic acids (FA with carboxyl group at both ends of the carbon chain), and this process occurs on ER.

2.2.3 Lipid trafficking

Lipid metabolism involves transport of different lipid structures, from FFA to complex lipids, at different levels. Transport of molecules occurs inside an organelle, between the cell organelles, between the cells and from acellular structures (as in endosperm of cereals) into living cells of the scutellum. Mechanisms behind lipid transport in plant tissues remain unstudied to a large extent, although some questions, mainly regarding processes in the living cells, have been addressed. In living cells, two major mechanisms for lipid movement have been proposed: vesicular and non-vesicular transport (van Meer et al. 2008; Benning 2008). The vesicular transport mechanism involves

lipid trafficking from plastid and ER membranes. Vesicle-inducing protein is believed to be involved in this process. PLs and FFAs released from TAG and PL for β-oxidation follow non-vesicular transport mechanisms. Flippases are proteins facilitating movements of PLs across membranes. There are ATP-dependent and ATP-independent flippases (Benning 2008; Samuels et al. 2008; Benning 2009). FFAs are believed to be transported into the peroxysomes by ATP-dependent proteins. Another mechanism of non-vesicular lipid transport through membrane contact sites has been proposed (Levine & Loewen 2006; Jouhet et al. 2007; Benning 2008). The activity of lipid transport proteins is the third method of non-vesicular lipid transport (Benning 2008; Xu et al. 2008; Chen et al. 2012). So far, this is the only known tool for extracellular lipid transport (Li-Beisson et al. 2010).

3 Results and discussion

3.1 Oil content and FA composition (Paper I)

Different approaches can be used to increase the oil content in oat and conventional breeding is one of them. In this approach, breeding a new variety involves using close relatives of the crop as donors of the trait of interest, such as high oil content or a particular FA. In paper I, I studied total lipid content and FA composition of 33 wild growing and 10 cultivated oat accessions to investigate the variation of these parameters in the existing germplasm. Samples of wild oat represented 13 species of different ploidy level (di-, tetra-, and hexaploid) while cultivated varieties were accessions of *A. sativa* L. (2n = 42). Significant differences in oil content were observed between accessions. Wild oat species contained up to 9.4% oil in the seeds with the mean value of 7.8%, which was significantly higher than the mean value for cultivated oat accessions (5.9%) (Leonova et al. 2008). Diploid oat species showed higher oil content compared to tetra- and hexaploid species.

Oat lipids were mostly composed of unsaturated oleic (18:1) and linoleic (18:2) and saturated palmitic (16:0) acids with low levels of stearic (18:0) and linolenic (18:3) acids (Zhou et al. 1999; Leonova et al. 2008). Hydroxy (avenoleic acid, 15-OH 18:1, and 7-OH 16:0) and epoxy (12,13-epoxy 18:1Δ9, 9,10-epoxy 18:1Δ12, and 9,10-epoxy 18:0) derivatives of FA were typically found at trace amounts in total oil but represented rather high percentages in certain lipid classes. There was a strong positive correlation between oil content and 18:1 amount while the level of 18:2 showed a negative correlation with both 18:1 and total oil amount. Similar patterns have been observed elsewhere (Sahasrabudhe 1979; Saastamoinen et al. 1989; Schipper et al. 1991) and it has been suggested (Grimberg 2009) that FAD activity remains constant over seed development while oil and 18:1 synthesis exhibit an exponential

increase. An alternative explanation suggested lipid turnover that occurred in oat seeds during maturation (Grimberg 2009).

Different lipid classes of oat oil and their FA composition were studied using thin layer chromatography (TLC). Each of nine spots obtained after lipid separation on TLC plate in neutral system was scraped off and analysed on GC. Analyses of sterol esters, however, were not included in the paper due to their non-FA nature. The major lipid class was TAG, followed by PL. This was an expected result since TAG is the major storage lipid and PL is the main membrane constituent. For each sample, TLC plates revealed six more spots clearly distinguishable from each other. One of them corresponded to FFA, a lipid class that does not occur in large quantities in cells due to its deleterious effect on cell membranes (Wu et al. 2006). In my study, the percentage of FFA in seeds of wild oat was 1,6% of total lipids, which was lower than the 2,4% observed in cultivars. The other five lipid classes were 1,2-DAG, 1,3-DAG, unknown lipid, TAG1, and TAG2.

Each lipid class demonstrated a specific FA composition which contributed to its separate distribution on TLC plate. Thus, avenoleic acid was mostly confined to PL, a membrane building compound. The presence of oxygenated FA in PL is rare since these FA are believed to have a harmful effect on the membrane functions. In my study, the PL fraction included various lipids with polar head group (phospho-, galacto-lipids, PE, PI – unpublished data). Avenoleic acid has been shown to be esterified to galactolipids (Hamberg et al. 1998), which are abundant in the plant cell membranes, particularly in the membranes of the photosynthetic apparatus (Dörmann & Heinz 2011). Avenoleic acid is structurally similar to ricinoleic acid in that it contains a homoallylic alcohol group at a position that can undergo desaturation (Hamberg et al. 1998). It is presumed that avenoleate is produced from linoleate by the action of putative linoleoyl 15-hydroxylase (Hamberg & Hamberg 1996a; 1996b). The other hydroxy FA found in oat are 7-OH 16:0, 13-hydroxy 18:2Δ9,11 and 9-hydroxy 18:2Δ10,12. The last two were not analyzed quantitatively in my study due to their sensitivity to acidic methylation applied to form methyl esters. In general, oxygenated fatty acid compounds (oxylipins) are believed to be involved in defense reactions initiated by different biotic and abiotic stresses (Kato et al. 1983; Kato et al. 1984; Hamberg et al. 2003).

Epoxy FA were observed in the unknown lipid, FFA (5,4-10%), TAG1, and TAG2, where I observed the highest levels of epoxidized FA (up to 30%). TAG1 and TAG2 showed much lower levels of 18:2 as compared to other lipid classes. Combination of high levels of epoxy FA and low levels of 18:2 in TAG1 and TAG2 suggested either that 18:2 was a substrate for enzymes

producing epoxy FA (peroxygenase, lipoxygenase, lipoperoxidase) (Hamberg & Hamberg 1996b; Meesapyodsuk & Qiu 2011) or that the substrate was 18:1, which instead of desaturation by FAD and producing 18:2 undergoes epoxygenation. In another study, 18:1 was shown to be a preferred substrate for AsPXG1 and AsLOX2 (peroxygenase and lipoxygenase identified in oat) (Meesapyodsuk & Qiu 2011). At the same time, other unsaturated FA (but only cis-forms), including 18:2, could also be oxidized by oat peroxygenase though with less efficiency. Saturated FA were shown not to be accessed by the enzyme thus excluding the possibility that 18:0 could be a precursor of oxygenated FA. Another study (Doehlert et al. 2010) indicated that in oat groats and oat flour stored at 37ºC, oxygenated FA were formed primarily from 18:2, although 18:1 was also found to be a substrate for producing epoxy FA.

There is also a controversy regarding the suitable substrate for oxygenases. It was concluded that hydrolysis of TAG to FFA is not required for FA oxidation (Doehlert 2010). However, a study has shown that, in vitro, only free FA were utilized by peroxygenase while acyl-CoA or FA esterified to PL were not suitable substrates (Meesapyodsuk & Qiu 2011). In my study, epoxy FA were found mostly in TAG1 and TAG2 while in all other lipid classes except PL, they were detected at about one-sixth the amount. 7-OH FA was only found in DAG1,2 and DAG1,3 and was not observed at detectable level in FFA. If we take into account the results from both studies above, this suggests three alternatives. First, FA could be oxygenated as free FA by peroxy- and lipoxygenase with subsequent esterification of produced epoxy and hydroxy FA to TAG and DAG. Secondly, they could be oxygenated as compounds of TAG and DAG. In this case, epoxy and hydroxy FA observed in FFA is either a result of lipolitic activity or of non-enzymatic oxygenation. Thirdly, FA could be oxidized on PC and incorporated into DAG via the enzyme PCDT, which transfers the head group from PC to DAG thus escaping production of FFA. Since free FA have detergent-like properties, their presence in a plant cell is deleterious and their utilization (by degradation via β-oxidation or incorporation into other lipids) is necessary for a cell to survive (Murphy 2005). This could explain the extremely low amounts of epoxy FA in PL.

The results presented in Paper I demonstrated variation among wild oat accessions in both oil content and oil quality. This variation can help to develop oils with diverse features and applications. The amount of omega 3 FA (α-linolenic acid), however, hardly showed any variation in the studied accessions. Oil content in wild oat species exceeded the one in cultivated oat by 2-3 %. Increasing omega 3 FA or oil contents by more than 5% would therefore require application of biotechnological rather than conventional breeding methods. However, if this approach is to be used, crossability of

cultivated oat with invasive weeds should be taken into consideration (EPOBIO 2011).

3.2 Lipid configuration in oat seed (Paper II)

As discussed in Chapter 2.1.2, different tissues of oat seed, i.e. embryo, scutellum, aleurone layer, and endosperm have an ability to accumulate reserve compounds (lipids, proteins, and carbohydrates) with the endosperm acting as the main storage site. Oat differs from other cereals in that its endosperm accumulates high levels of lipids (Youngs et al. 1977; Zhou et al. 1999; Banas et al. 2007). Elevated lipid content in the seeds of high-oil oat varieties, as compared to medium-oil varieties, is due to more intensive lipid accumulation in the endosperm rather than the embryo (Banas et al. 2007).

Oil in most plant seeds exists as discrete oil bodies – droplets of TAG surrounded by PL monolayer incorporating proteins, oleosins and caleosins (Tzen & Huang 1992). However, recent studies have demonstrated that at early stages of oat grain development lipids in the endosperm existed as oil bodies, while after mid stage of seed maturation these oil bodies were fused into smears (Banas et al. 2007).

The inability to detect distinct oil bodies in oat endosperm was interpreted in an earlier report as an artefact of sample manipulation or as an indication of the absence of oil in the endosperm (White et al. 2006). We conducted this study to further investigate the structural peculiarities of lipid accumulation in oat seed.

The seeds of two oat cultivars, medium- and high-oil (cv. Vital and cv. Matilda), were analyzed at two stages of development and at maturity, using different staining (M-A-S, TB, SB) and microscopy (LM, SEM, TEM) methods. Our results were consistent with the conclusions of Banas et al. (2007), confirming the change of lipids configuration in the oat endosperm during seed development. Oil bodies were formed in early stages of grain development in all tissues. In the endosperm, they lost their discrete appearance and turned into fused smears at the seed maturity. Lipids were present as oil bodies in living cells (i.e. embryo, scutellum, and aleurone layer) throughout the seed maturation. This observation did not support interpretation of their appearance as an artefact of sample preparation.

Two types of oil body-associated proteins, oleosins and caleosins, likely act as stabilizers of the oil body, preventing its coalescence. Such action helps enhance the accessibility of TAG to lipid degrading enzymes during seed

germination (Tzen & Huang 1992; Ross et al. 1993; Leprince et al. 1998; Murphy et al. 2001; Liu et al. 2009).

Therefore, our hypothesis was that oat endosperm has a significantly lower amount of oleosins compared to living tissues of oat seed where such oil body fusion does not occur. To test this hypothesis, oil from embryo-scutellum and endosperm of cv. Matilda seeds was extracted and proteins associated with oil bodies were purified. SDS-PAGE separation of these proteins demonstrated the presence of three protein fractions, i.e. 14, 16, and 28kD. Judging by their molecular weight and oil body association, the 14 and 16kD fractions were most probably isoforms of oleosins (Huang 1992) and 28kD fraction is caleosin (Chen et al. 1999). The third oil-body associated type of protein, steroleosin (Lin et al. 2002), was not observed in my study. While the 28kD fraction was more pronounced in endosperm samples, the oleosins showed remarkably darker bands in embryo-scutellum samples than in endosperm samples. Higher levels of caleosins in the endosperm fraction could originate from oil bodies of the aleurone layer, assuming that cells of the aleurone layer contained more caleosins than oleosins. These results were corroborated in experiments with immunolocalization by antibodies against the 16 kD protein. This protein was predominant in the embryo, scutellum and aleurone layer but exhibited a significantly lower concentration in the starchy endosperm (Heneen et al. 2008).

TEM conducted on oat seed tissues during early development stages revealed both the presence and the lack of PL membranes coating oil bodies in endosperm cells. Variability in boundary appearance was not observed in cells of embryo, scutellum, and aleurone layer (Heneen et al., 2008). In the endosperm, I observed oil bodies with fragments of uncoated border, were in turn associated with the merging of oil bodies with adjacent lipids. This supported the hypothesis of non-concurrent biosynthesis of TAG and PL membranes (Frandsen et al. 2001).

At the same time, oil bodies in non-matured endosperm cells were abundant in regions rich in ER and ribosomes, indicating the involvement of these organelles in oil body synthesis. This observation supported the hypotheses that (a) the process of oil body synthesis is ER dependent and (b) biosynthesis of TAG and PL membrane occurs concurrently (Hsieh & Huang 2004). Thus, our observations support both hypotheses, suggesting simultaneous presence of concurrent and non-concurrent synthesis of TAG and PL membrane, corresponding to ER-dependent and ER-independent oil biogenesis (Huang 1992).

3.3 Lipid mobilization (Paper III)

Utilization of compounds stored in the endosperm requires the activity of a range of hydrolysing enzymes which are able to convert large molecules of reserved carbohydrates, proteins and TAGs into small units deliverable to the growing embryo. The enzymes necessary for the mobilization of reserves can only be produced in the living cells. In case of lipids, these enzymes are primarily (a) lipases which destroy the ester bond between FA and glycerol moieties and (b) secondly enzymes of FA β-oxidation (Clarke 1983; Graham 2008). Endosperm cells of oat seed go through programmed cell death upon seed maturation (Young & Gallie 2000) and thus lack organelles, peroxysomes and glyoxysomes, to hostthe enzymes for β-oxidation of FAs. Therefore, even if lipases can be secreted into the endosperm from living cells of the surrounding aleurone layer and scutellum, the freed FA has to be delivered to living tissues, most probably scutellum and embryo, for FA β-oxidation. That means that rather large FA molecules should be transported from the inner endosperm regions to the scutellum epithelium cells and cross both their double membrane and cell wall. Such a process appears biochemically challenging. The phenomenon of oil bodies' coalescence in the endosperm tissue discussed in the previous chapter seems to present another obstacle in efficient lipid utilization since it minimizes the contact area between substrate and enzymes. Endosperm cells cannot carry out the reactions needed for FA utilization and also lack discrete oil bodies' structures. This combination of features is remarkable since it questions the importance and meaning of oil reserves in oat seed.

To follow the fate of oil reserves in oat endosperm, seeds of high- and medium-oil cultivars, Matilda and Freja, were imbibed in water and allowed to germinate in darkness (to eliminate the contribution of photosynthesis in embryo growth) for 10 days. The amount and quality of oil and starch reserves were analyzed in separated embryo+scutellum and endosperm tissues at several time points, 0, 1,2, 4, 7, and 10 days after imbibitions (DAI).

The results of this study revealed the steady decrease in total oil amount in the endosperm. The proportion of TAG fell considerably already during the first two days of germination while that of FFA increased (Leonova et al. 2010). However, the increase of FFA content in germinating oat seed was lower than the amount of released FFA from TAG degradation. FA composition of endosperm, scutellum, and embryo lipid classes were analysed by GC. These analyses showed a gradual change in FFA quality so that by the 4^{th} DAI the FA composition of embryo and scutellum FFA resembled almost completely the FA composition of endosperm TAG. However, at this time point, the 18:1/18:2 ratio of the endosperm FFA, was lower than TAG and more similar to that of

endosperm PL. This can be explained, firstby 18:1 affinity of lipases, or second and more likely, by the presence of two types of lipases acting on endosperm TAG (Fig. 5). Lipases released into endosperm from the aleurone layer are specific to PL but can also utilize TAG when PLs have been degraded. The FFAs coming from PL degradation are spatially distant from the absorptive scutellum and thus are accumulating in endosperm, thereby influencing its FFA composition. Lipases secreted from scutellum have preference to TAG; products of their activity are efficiently taken up by the scutellum, thus making the FA composition of scutellum FFA similar to that of endosperm TAG.

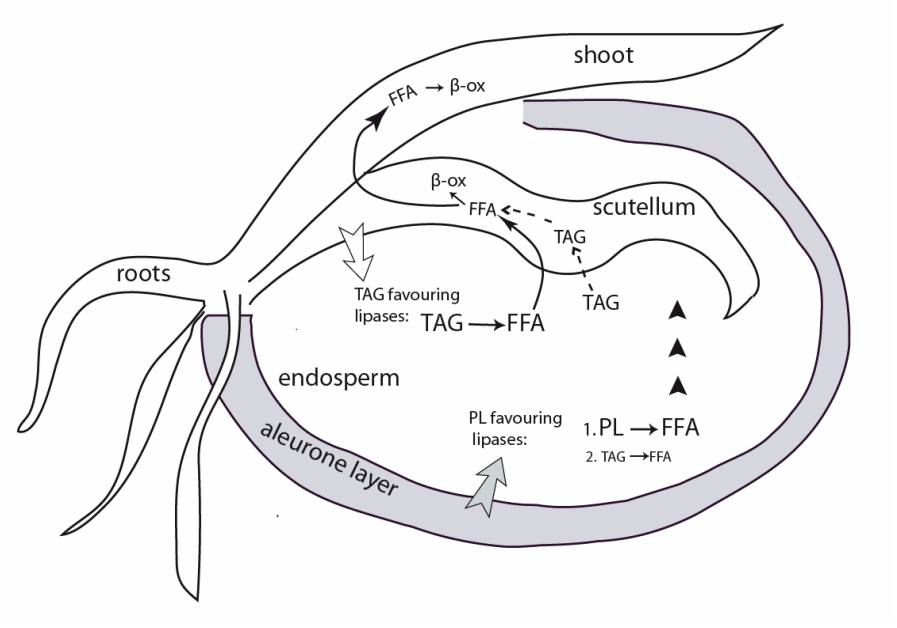

Figure 5. Suggested pathways of lipid mobilization in germinating oat seeds. Aleurone layer secretes PL-favoring lipase (grey arrow), which can also use TAG as substrate after PL reserves are depleted; scutellum secretes TAG-favoring lipases (white arrow). FFA from TAG near the scutellum are absorbed by scutellar epithelium cells immediately and further transported either as FFA or/and as sugars (big arrow heads), FFA from PL and TAG near the aleurone layer are absorbed by the scutellum later in the germination process (small arrow heads). A less likely alternative is direct uptake of TAG by scutellum and its immediate degradation to FFA (dashed arrows).

Our study suggested that lipids can be absorbed by scutellum together with the products of carbohydrate and protein degradation (Edelman, Shibko, & Keys 1959; Walkersmith & Payne 1984). This was indicated by a decrease of TAG in the endosperm and accumulation of FFAs in the scutellum, which were

almost identical in their composition to that of endosperm TAG. This pattern resembled that of germinating oil palm seed, where an organ called the haustorium absorbs nutrients, including FFA, from the endosperm (Boatman & Crombie 1958).

According to data obtained by TEM, during germination the fused oil in the oat endosperm changed into oil droplets in areas close to the aleurone and the scutellar epithelium. However, these oil droplets are unlikely to be true oil bodies surrounded by PL membrane with oleosins since their appearance suggested absence of well-defined boundaries. I observed a close contact between oil droplets of endosperm and epithelial cells of. Thus, both our biochemical and TEM analyses of germinating oat seed suggested that oat scutellum delivers FFA from endosperm TAG to the growing tissues. This delivery can operate either with intact FFA or sugars obtained in FA β-oxidation process. The enzymes for FA degradation (via β-oxidation and glyoxylate cycle) have been identified in the haustorium of oil palm (Oo & Stumpf 1983; Alang, Moir, & Jones 1988) and the scutellum of maize (Oaks & Beevers 1964) and rice (Okamoto et al. 1982).

Our results show that the main difference in TAG mobilization between high- and medium-oil oat cultivars cultivars lies in the rate of FFA transfer from endosperm to the scutellum and the capacity to accumulate starch in scutellum, which are both higher in high-oil cultivars. In particular, the FFA transfer rate in high-oil oat cultivars can be 28% higher than in medium-oil cultivars (Leonova et al. 2010). This indicates that high-oil oat has developed more efficient machinery not only for lipid biosynthesis but also for its utilization during seed germination.

4 Towards oat with increased oil content and improved FA composition

4.1 Oil content

Modern society needs renewable sources of energy and the scientific community has been active in finding practical solutions of this problem (Benning & Pichersky 2008; Radakovits et al. 2010; Carlsson et al. 2011). Plants exhibit a sustainable and potentially endless source of energy, which may address the energy requirements of the growing world population. This assumption is contingent upon development of new plant varieties which can accumulate more oil at the expense of carbohydrates, which are inferior to oils in respect to their capacity to accumulate energy. Such switching in accumulation pathways can be achieved through a modification of a chemical mechanism controlling carbon allocation (Grimberg 2009).

Oat could be one of the promising candidates for such research since it is already rich in oil and has a number of agronomic and nutritional advantages compared to other crops. Diploid oat species showed higher oil content compared to tetra- and hexaploid species (Leonova et al. 2008), and thus are good donors for this trait. Crossing diploid oat with existing elite hexaploid cultivars, however, is difficult and usually results in a large production of non-viable seeds (Rodionova et al. 1994). To the best of my knowledge, the highest oil content ever reported in existing cultivars and wild species of genus *Avena* is 13% (Lehtinen & Kaukovirta-Norja 2011). To use oat as an oil crop an increase in seed oil content to at least 20% would be required (Frey & Hammond 1975). There has been some success in raising oat oil content by recurrent selection but a selection-driven increase in the amount of oil (by 18%) was associated with a decline in seed size, which led to a decrease in total yield (Holland et al. 2001). These considerations warrant the use of non-conventional breeding techniques for developing oat as an oil crop.

Over-expression of *DGATI* did not compromise seed size while increasing oil content in Arabidopsis and *B. napus*. In these experiments, an increase in total oil content was accompanied by an increase in both seed size and weight (Jako et al. 2001; Sharma et al. 2008). The DGATI role in TAG assembly was confirmed by the observations of increased DGATI activity in microsomal preparations and decreased ratio DAG/TAG in transgenic seeds. Further analyses of these transgenic lines have identified hormonal and transcriptional changes that are not confined to TAG biosynthesis (Sharma et al., 2008), thus indicating the importance of other metabolic pathways in seed oil accumulation. Similar increases in both oil content and seed size were obtained in transgenic Arabidopsis and *B. napus* plants with down-regulated mitochondrial pyruvate dehydrogenase complex (mtPDC) kinase. mtPDC converts pyruvate into acetyl-CoA, a precursor for FA biosynthesis. In turn, mtPDC kinase suppresses mtPDC and its down-regulation causes an increase in the oil synthesis and seed (Zou et al. 1999; Marillia et al. 2003). Transcription factor WRINKLED1 (WRI1) has been demonstrated to have regulatory action towards mtPDC kinase (Baud et al. 2007a; 2007b), which opens a possibility to manipulate the activity of mtPDC kinase and therefore the oil biosynthesis at the level of transcription factors.

Variety, climate conditions, level of nitrogen in the soil, and geographic location are important factors influencing oil content in oat seeds. For instance, the positive effect of low growing temperatures on oat seed oil accumulation has been demonstrated (Beringer 1971; Saastamoinen et al. 1989).

Investigations in oat lipid metabolism have shown that oil accumulation in a high-oil variety (cv. Matilda) occurred in the first days after onset of seed formation and continued through the whole period of seed development. In a medium-oil variety, however, oil accumulation was much less intense in the first stages of seed formation and further decreased in the late stages (Banas et al. 2007) (Banas et al. 2000; Grimberg 2009). A similar pattern has been previously shown in rapeseed (Chia et al. 2005) and Arabidopsis (Baud et al. 2002). Maintaining high expression of genes responsible for oil biosynthesis would be desirable in order to increase oil accumulation. In this context, identification of transcriptional factors controlling the expression of these genes is crucial Several candidates, besides the above mentioned WRI1, have been suggested: GLABRA2, LEAFY COTYLEDON1 (LEC1), LEC2, ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 from Arabidopsis reviewed in (Santos-Mendoza et al. 2008), DNA BINDING WITH ONE FINGER4 (DOF4) and DOF11 (Wang et al. 2007).

So far, the carbohydrate-lipid carbon flux regulator is still undergoing the process of identification, and other ways to increase oil in crops have been

utilized. There has been activation of FA biosynthesis via overexpression of ACCase and enhancing TAG assembling via either overexpression of enzymes involved in TAG synthesis (Andrianov et al. 2010) or downregulation the enzymes involved in TAG breakdown (Slocombe et al. 2009).

4.2 FA composition

Plants can be viewed as perfect chemical factories that are able to produce an enormous variation of FA in environmentally friendly way. Unfortunately, production of some FAs which have value for humans is often restricted to one or few non-oil crop species. Examples include jojoba accumulating wax esters (Al-Hamamre 2013), macadamia trees producing oil rich in 16:1 FA (Griel et al. 2008), and *Euphorbia lagascae* Spreng exhibiting high level of epoxy 18:1 (vernolic acid) (Krewson & Scott 1966). Such FA have been much cheaper to produce at ordinary chemical factories. Utilization of genetic engineering techniques has made it possible to increasingly shift production of FAs of interest back to the crop fields, using genetically modified races of plants with naturally high oil content. An example of such trend is a line of crambe with high erucic acid content, developed by biotechnological methods (Li et al. 2012).

Increased consumption of omega 6 FA and low intake of omega 3 is common problem in the diets of modern humans, shown to cause different health disorders such as obesity, vascular diseases, arthritis, autoimmune and inflammatory diseases (Simopoulos 2008). So far, the only substantial source of omega 3 FA is fish oil. In turn, fish obtain the omega 3 FA from marine microorganisms which possess highly active enzymes for elongation (FAE) and desaturation (FAD) of FA (Damude & Kinney 2007). Such enzymes do exist in other species. By infecting rapeseed explants with *Agrobacterium* carrying *FAD*s from fungus *Mortierella alpine* (genes coding delta6 and delta12 FAD) and *Brassica napus* (delta15 FAD), rapeseed lines with considerable accumulation of omega 3 stearidonic FA in the seeds have been obtained (Ursin 2003). Rice with elevated levels of alpha-linolenic acid has been obtained via *Agrobacterium*-mediated transformation with *FAD3* cloned from soybean (Anai et al. 2003).

Increase in the concentration of omega 3 FAs in oat seeds would help mitigate the FA imbalance of modern diets. Presently, oat seeds exhibit only trace amounts of omega 3 α-linolenic FA. It follows that introduction of *FAD* and *FAE* into oat genome and synthesis of respective enzymes would balance the proportion of omega 3 and omega 6 FA, improving the nutritional characteristic of oats.

4.3 Oat transformation

Genetic engineering is a precise and effective method to modify an unfavourable trait or introduce a new trait into a crop with a limited gene pool for breeding. Oat is a good example of such species since its natural gene pool does not contain sufficient genetic variability in oil content and undesirable fatty acid (FA) composition, namely unbalanced ratio of omega3/omega6 FA, which have been previously studied (Leonova et al. 2008). Using genetic engineering to deliver a gene of interest into a plant genome often requires (a) a well established protocol for in vitro shoot regeneration; (b) a well working transformation protocol; (c) use of a selection marker for facilitating the growth of transformed cells while suppression of non-transformed cells.

In vitro shoot regeneration means to culture a part of plant tissues or organ (called explants) on a medium containing nutrients and photohormones under sterile conditions. In the presence of phytohormones, usually auxin 2,4-D for cereal crops, the explant starts to divide and form undifferentiated cells, called callus. Such calli would have capacity for somatic embryogenesis or regeneration via organogenesis. After the calli have reached the phase to be able to regenerate plantlets, they are moved to a medium containing different phytohormones for promoting shoot formation. Conditions of tissue culture can be crucial for *Agrobacterium*-mediated transformation (Birch 1997; Riva et al. 1998).

Positive selection systems are used in selection of putatively transformed cells. The selective agents can be antibiotics (kanamycin, hygromycin) or herbicides (phosphinotricine, PPT). However, presence of genes coding for antibiotic or herbicide resistance in genetically modified commercial crop varieties may not be desirable in cultivated crops due to the risk of horizontal gene transfer.

Two strategies have been developed to solve this problem: 1) excision of the selectable genes from the genome of GM plant by various methods, reviewed elsewhere (Barampuram & Zhang 2011) and 2) use of other types of selection systems (Kaeppler et al. 2000; 2001; Penna et al. 2002; Aragao & Brasileiro 2002; Scheid 2004; Penna & Ganapathi 2010; Duan et al. 2012), such as mannose selection system. In this system, the selectable marker is either a gene *manA* (or *pmi*) gene coding phosphomannose isomerase (PMI) or *P6MR* encoding mannose-6-phosphate reductase. Both are able to convert mannose-6-phosphate, a sugar non-accessible for plants, into substances, which can be utilized by plant cells as carbon source. In case of PMI that is fructose-6-phosphate (Joersbo & Okkels 1996; Reed et al. 2001) and in case of P6MR that is mannitol (Song et al. 2010). The PMI system is known longer than P6MR and has been used in transformation of a number of species, such as

sugar beat (Joersbo et al. 1998), maize (Wang et al. 2000; Negrotto et al. 2000; Wright et al. 2001), sorghum (Gao et al., 2005), barley (Reed et al., 2001), wheat (Wright et al. 2001), durum wheat (Gadaleta et al. 2006), pearl millet (O'Kennedy et al. 2011), and rice (Lucca et al. 2001; Duan et al. 2012).

For oat, it is particularly important to avoid introducing genes coding for antibiotic resistance as oat is easily to be crossed with invasive weeds from genus *Avena* (if both are of the same ploidy level) (EPOBIO 2011).

4.3.1 Non-*Agrobacterium*-mediated oat transformation

The first successful transformation of oat was reported in 1992 (Somers et al. 1992). Calli derived from immature embryos (IME) were bombarded with DNA-coated tungsten particles and putatively transformed cells were recovered on PPT selection medium. The incorporation of the *bar* gene coding for phosphinotricine acetyl transferase in the genome of regenerated plants was confirmed by PCR and Southern blot. After publication of this result, many modifications have been made for improving oat transformation efficiency, including usage of various explant types, selection systems, promoters and genotypes (reviewed in Molnar et al. 2011). However, the biolistic transformation method is not desirable as it can introduce gene silencing due to a high copy number of transgene integration (Pawlowski et al. 1998; Pawlowski & Somers 1998; Svitashev et al. 2000). This problem has also been found in other cereal species (Register et al. 1994; Kohli et al. 1999). Development of an alternative method of DNA delivery is therefore needed. One good option would be *Agrobacterium*-mediated transformation which often results in low copy numbers of transgene integration (Repellin et al. 2001; Sharma et al. 2005).

4.3.2 Establishing *Agrobacterium*-mediated oat transformation (Paper IV)

In my study, I tried to develop a protocol for *Agrobacterium*-mediated oat transformation, based on studies reporting successful transformation in cereals (Danilova 2007; Dahleen & Manoharan 2007; Gasparis et al. 2008; Bartlett et al. 2008; Jones & Shewry 2009). My study did not succeed in transforming oat although I tested multiple transformation protocols with different cultivars, types of explant, selection systems, binary vectors, *Agrobacterium* strains, infection conditions, and co-cultivation and regeneration media. I also tested the only published protocol of reportedly successful *Agrobacterium*-mediated

oat transformation (Gasparis et al. 2008). All but one parameter of the protocol were carefully repeated, the only remaining parameter constraint among tests being the binary vector. Although I produced a number of regenerates which grew under kanamycin pressure, no confirmation of transformation event was obtained, meaning that these plantlets were non-transgenic selection escapes. My failure to transform oat might indicate that the construct was of crucial importance for successful oat transformation. It also could be that the published description of the procedure did not provide an adequate description of one or several important steps of the protocol.

Oat has endogenous GUS activities. For evaluation the transgene GUS activities, a method to remove this internal GUS signal must be developed. I compared different protocols for GUS staining for suppressing the endogenous GUS expression in oat tissues. I found that pH 7.5 and lower gave blue staining of scutellum of non-transformed ME and that this colour became stronger after seven days. pH 7.8 was efficient in muting endogenous GUS even if samples were kept in the solution over more than six months. I also tested addition of methanol to avoid false positive GUS staining result with the methanol concentrations of 0-50%. At pH 6.0, addition of 20% methanol did not have any effect on GUS staining, but at pH 7.8, 20% methanol could mute GUS signal from the wild type and these conditions were used for further analyses.

I tested various factors that may affect the infection process. Of which, pretreatment of explants with low temperatures (+4 or +10ºC) seemed to promote the transient GUS expression although temperature of 19-25 ºC has been previously reported as optimal for *Agrobacterium* infection (Fullner & Nester 1996; Salas et al. 2001). Another factor associated with strong transient GUS expression in my trials was the use of infection media with high sugar content.

I did not observe the positive effects of desiccation reported in earlier studies (Urushibara et al. 2001; Cheng et al. 2003). Addition of antioxidant compounds such as silver nitrate, ascorbic acid, and cysteine into CI and co-cultivation media was also evaluated for improving transient GUS expression and my results suggested that desiccation and antioxidant addition appear to be promising but more studies are required.

Since optimal conditions for tissue culture is the first and crucial step toward successful transformation, I carried out a number of experiments where some important factors affecting in vitro shoot regeneration were evaluated, such as genotype, plant growth regulators (PGR), in the regeneration medium, and type of explant. Mature embryos (ME) and leaf base (LB) from cultivars Freja, Matilda, Kerstin, and Vital were tested. I compared several variants of hormones, including NAA+BAP+kinetin, ZR, BAP, kinetin, IAA+BAP,

zeatin, and TDZ. Calli derived from LB of Freja and Kerstin showed 70-100% regeneration frequency on all PGR media tested. ME-derived calli in general were less efficient in regeneration. The best result obtained was 80% shoot regeneration from ME derived calli from Kerstin on IB. Vital had poor regeneration frequency as compared to other tested cultivars. However, it was possible to increase the regeneration frequency up to 90%, using LB cultivated on NBK, or ZR, or TDZ+BAP from plants grown in the biotron, indicating the same cultivar performed differently depending on donor plants' growing conditions. This conclusion supports the results reported earlier (Vasil 1987).

The optimum level of selective agents for a particular genotype is another requirement for successful transformation. Using two oat cultivars, Vital and Matilda, I tested four selection agents, kanamycin, mannose, hygromycin and phosphinothricin in order to find out appropriate selection pressure in each selection system. The results showed that 50mg L^{-1} kanamycin, 1% mannose, 25mg L^{-1} hygromycin and 2.5mg L^{-1} phosphinothricin appear to work well for oat transformation.

The results of my transformation trials reconfirmed the observation that high regeneration percentage does not necessarily secure successful transformation. It appears that a suitable combination of *Agrobacterium* strain, binary vector, selectable agent, explant type, and specific conditions for infection and regeneration of infected explants is needed for successful oat transformation.

5 Conclusions and future prospects

5.1 Conclusions

In this study, I investigated various aspects of lipid metabolism in oat seeds.

1. Wild growing species of genus *Avena* possess higher oil amounts and wider variation of lipid classes and FA composition compared to cultivated oat. The studied species had low content of omega 3 FA (2% or less) and lacked variation in the amount of this FA. Application of genetic engineering may help increase the amount of omega 3 FA and thus balance the omega 3/omega 6 proportion in oat seed.
2. Ultrastructural studies on oat seeds with several staining methods revealed the fusion of oil bodies in the starchy endosperm upon seed maturation. Oil bodies in the embryo and aleurone layer kept their discrete appearance. Such merging of oil bodies is probably promoted by lower levels of oleosins in the endosperm, as compared to the living tissues.
3. During germination, oat seeds do make use of the lipid reserves in the starchy endosperm. Scutellum absorbs lipids either as FFA, or as intact TAG (less often) and delivers them to the growing embryo. TEM analyses demonstrated a close contact between endosperm oil droplets and scutellar epithelium thus confirming absorptive function of scutellum, previously shown by biochemical data.
4. Trials to transform oat via *Agrobacterium* did not lead to the confirmed integration of the transgene into oat genome even though several plants were regenerated on selection media. Apparently successful oat transformation is a product of a unique combination of *Agrobacterium* strain, binary vector, selectable agent, explant type, and specific conditions for infection and regeneration of infected explants. In my study, I succeeded in identifying a genotype which is highly responsive to tissue culture. I demonstrated that manipulating growth regulators and explant type are

ways to improve the performance of a poorly regenerating genotype *in vitro*. Donor plants grown under optimal conditions provide explants with improved performance in tissue culture.

5.2 Future prospects

Oat has a considerable nutritional and economic potential as an oil crop. Although conventional breeding offers good possibilities to improve oat characteristics in terms of oil content and quality, this method is time consuming and labour intensive. Alternatively a biotechnological approach can be used, which opens endless possibilities in manipulating groat parameters including oil composition and content.

Currently, application of biotechnological approaches to improve nutritional qualities of oats faces two challenges. The first is finding key regulators of enzymes involved in oil biosynthesis. Studies have documented the presence of mechanisms responsible for high production of oil at early stages of seed development. Maintaining such biochemical activity over a larger part of seed maturation process may provide means to boost oil yield. Biochemically, this would imply a redirection of carbon flow from starch to lipids. Among the tools to detect the genes involved in lipid biosynthesis are (a) QTL analyses for groat oil content, (b) identification of the expressed genes and their sequencing, and (c) downregulation or overexpression of candidate genes.

The second challenge is development of a reliable *Agrobacterium*-mediated transformation protocol, which would be superior to current prevailing transformation via microprojectile bombardment. A breakthrough in developing a working *Agrobacterium*-mediated transformation protocol for oats will be contingent upon progress in understanding the mechanisms lying behind the delivery and incorporation of transgenes into the oat genome.

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High oil content is one of the characteristic features of oat grain which makes oat a potential oil crop. Oil content and its lipid classes and fatty acid composition of various wild growing oat species, distribution of oil in the grain and particularly in the starchy endosperm, and mobilization of lipid reserves in germinating oat seeds have been studied. Efforts on developing Agrobacterium-mediated oat transformation and improving oat regeneration protocol were also undertaken.

Svetlana Leonova received her graduate education at the Department of Plant Breeding, SLU, Alnarp and her MSc at the Herzen State Pedagogical University, Saint-Petersburg, Russia.

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