# Carbon Partitioning Between Starch and Oil in *Avena sativa* (Oat) and *Arabidopsis thaliana*

## Method Development and Biochemical Studies Paving the Way for Future Oil Crops

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Cover: Seeds and leaves of *Avena sativa* (oat).

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## Abstract

In recent years, the demand for plant-derived oils as renewable alternatives to fossil oil has increased due to the rising cost of petroleum and the increased concern about the environment. However, the supply of vegetable oils today relies upon only a few crops with low potential for further increases in oil yields. Redirection of carbon flux from starch to oil in the cereal seed can provide for new high-yielding oil crops as a sustainable alternative to fossil oil to meet the increased need for plant oils. Increased knowledge of oil biochemistry and the regulation of carbon partitioning between different storage compounds in seeds is therefore of crucial importance for the development of such novel oil crops in the future.

In a study to determine if the future environment with elevated atmospheric $CO_2$ can be expected to alter the partitioning of carbon into oil in seeds, it was found that elevated $CO_2$ induced a much higher starch accumulation in leaves of *A. thaliana* whereas seed oil content was unaffected. However, leaf lipid metabolism was altered in an elevated $CO_2$ environment and changes were targeted to the lipids in the photosynthetic membranes of the chloroplasts.

Among the cereals, oat is unique in storing high amounts of oil in the endosperm. The deposition of storage compounds during seed development and their mobilization during germination was investigated. There were large differences in partitioning of carbon between oil and carbohydrate reserves between oat cultivars having different oil content, both during seed development and germination. The data suggests that oat is suitable as a model plant for oil synthesis in the cereal endosperm to find the metabolic switches for carbon partitioning between starch and oil.

An *in vitro* liquid culture for oat seeds on detached panicles was established to mimic seed development and cultivar differences in storage accumulation from anthesis to maturity *in planta*. The *in vitro* system was used in preliminary stable radioactive isotope labeling experiments that confirmed the cultivar differences in carbon partitioning into oil observed *in planta*.



*Keywords: Arabidopsis thaliana*, *Avena sativa*, carbon partitioning, cereals, elevated $CO_2$, endosperm, germination, oat, oil crop, starch, triacylglycerol



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# 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 Ekman Å., Bülow L., Stymne S. 2007. Elevated atmospheric $CO_2$ concentration and diurnal cycle induce changes in lipid composition in *Arabidopsis thaliana*. *New Phytologist* (174) 591-599.

II Banas A., Debski H., Banas W., Heneen W., Dahlvist A., Bafor M., Gummeson P-O., Marttila S., Ekman Å., Carlsson A., Stymne S. 2007. Lipids in grain tissues of oat (*Avena sativa*): differences in content, time of deposition, and fatty acid composition. *Journal of Experimental Botany* (58:10) 2463-2470.

III Grimberg Å.*, Leonova S. Stymne S., Carlsson A. Mobilization of lipid reserves during germination in two cultivars of oat (*Avena sativa*) having different amounts of endosperm oil. (Manuscript).

IV Ekman Å., Hayden D.M., Dehesh K., Bülow L., Stymne S. 2008. Carbon partitioning between oil and carbohydrates in developing oat (*Avena sativa* L.) seeds. *Journal of Experimental Botany* (59:15), 4247-4257.

Appendix A. Definitions of developmental stages of oat seeds *in planta*.

Papers I, II, and IV are reproduced with the kind permission from the publishers.

**Grimberg Å., maiden name Ekman Å.*

The contribution of Åsa Grimberg to the papers included in this thesis was as follows:

I Planned and performed the experimental work and the evaluation of data, wrote the paper together with co-authors.

II Performed part of the experimental work and the evaluation of data, took part in the writing of the paper.

III Planned and performed a large part of the experimental work and the evaluation of data, wrote the manuscript together with co-authors.

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

# Abbreviations

| | |
|---|---|
| 18:1 | 18 carbon chain fatty acid with one double bond |
| ACC | acetyl-CoA carboxylase |
| ACP | acyl carrier protein |
| ADP | adenosine diphosphate |
| AGP | adenosine diphosphate-glucose pyrophosphorylase |
| ATP | adenosine triphosphate |
| CoA | co-enzyme A |
| DGAT | diacylglycerol acyltransferase |
| DGDG | digalactosyldiacylglycerol |
| dpa | days post anthesis |
| dw | dry weight |
| ER | endoplasmatic reticulum |
| EST | expressed sequence tag |
| FA | fatty acid |
| FAME | fatty acid methyl ester |
| FAS | fatty acid synthesis |
| FFA | free fatty acid |
| Frc | fructose |
| G3P | glycerol-3-phosphate |
| GC | gas liquid chromatography |
| Glc | glucose |
| LPAT | lysophosphatidyl acyltransferase |
| MGDG | monogalactosyldiacylglycerol |
| ODN | oligodeoxynucleotide |
| P | phosphate |

| | |
|---|---|
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PEP | phosphoenolpyruvate |
| PG | phosphatidylgycerol |
| st | developmental stage |
| TAG | triacylglycerol |
| TLC | thin layer chromatography |
| UDP | uridine diphosphate |

# 1 Introduction

Plant oils derived from oilseed crops represent an important agricultural commodity that is used primarily for food and feed purposes today. In recent years, the demand for plant-derived oils as renewable alternatives to fossil oil has increased due to the rising cost of petroleum and the increased concern about the environment (Dyer *et al.*, 2008). Plant derived oils are the agricultural products that chemically are most similar to fossil oil and thus could be used for both fuel production and in the chemical industry (Durrett *et al.*, 2008; Dyer *et al.*, 2008; Nikolau *et al.*, 2008). Compared to the conversion of carbohydrates into ethanol, the conversion of plant oil (triacylglycerol; TAG) into biodiesel (*i.e.* fatty acid methyl esters; FAMEs) is much more energetically favorable (Hill *et al.*, 2006) and is therefore a more sustainable alternative to fossil oil. Even though the combustion engines of cars will probably be replaced by electrical motors in the near future, cargo vehicles like trucks and boats will probably continue to use diesel for a long time. This diesel can, to a significant proportion, be replaced by plant derived oils if the supply of feedstock is increased.

The world production of vegetable oils increased with 60% between 1997–2007 (Fig. 1, FAOSTAT, 2008). However, the supply of vegetable oils today relies upon only a few crops; palm oil (*Elaeis guineensis*), soy bean (*Glycine max*), rape seed (*Brassica napus*), and sunflower (*Helianthus annuus*), which in 2007 accounted for 83% of total world production (FAOSTAT, 2008). Oil palm (50% of oil in the fruit) is a high yielding crop (five times more than rape) that requires relatively low input of energy and fertilizers. However, it can only be grown in a small geographical area close to the equator. Soy bean (20% of oil in the seed) is an environmentally attractive oil crop due to its ability to fix atmospheric nitrogen and thus reduced need for nitrogen fertilizers. Nevertheless, soy bean is mostly appreciated for its high quality protein content that has a higher economic value than the oil.

Rape seed (45% of oil in the seed) is the annual crop with the highest oil yield in Northern Europe. However, rape seed requires large input of fertilizers and pesticides and also needs to be rotated and can only be grown each fifth year on the same field. Due to these reasons, the oil production from these crops is not expected to increase in accordance with the increased demand. The restricted supply of feedstock due to the limited amount of oil crops is therefore now one of the biggest challenges in plant oil production in the future (Thelen & Ohlrogge, 2002b; Durrett *et al.*, 2008).

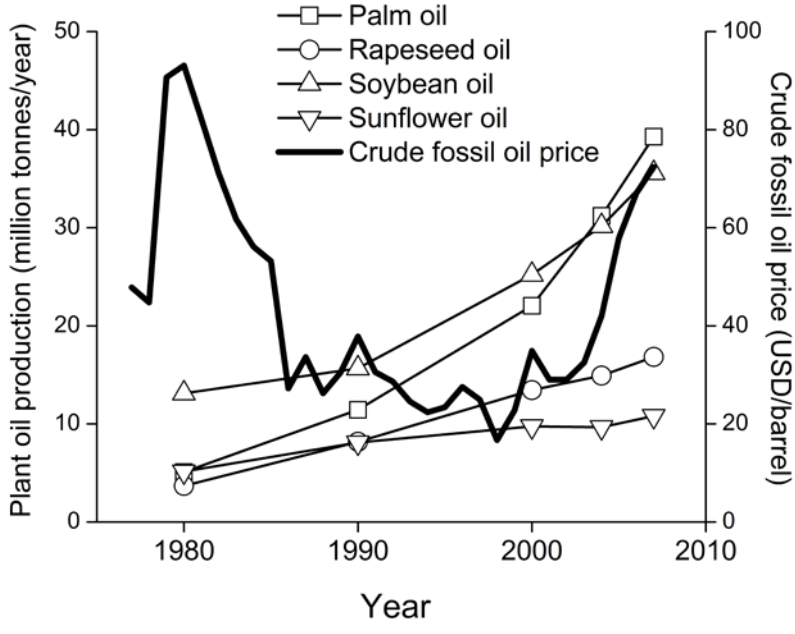



*Figure 1.* Yearly global production of plant oils 1980-2007 from the four most common oil crops in the world (FAOSTAT, 2008) and crude fossil oil prices 1976-2007 given in 2007 currency (BP, 2008).

The rapid unraveling of gene functions and biochemical pathways and the advancement of Systems Biology technologies open the opportunities to reprogram plant cell metabolism. One way to generate new oil crops that are economically viable is therefore to redirect carbon flux within plants from carbohydrates to oil. If this could be achieved in the endosperm cells of cereal seeds it would enhance the oil levels to give an oil productivity exceeding that of rape seeds since cereals are, in general, much higher yielding crops. For example, if wheat yielding approximately 6 tonnes $ha^{-1}$

(Northern Europe) could be converted to an oil crop with 30% oil (w/w), this oil-wheat would yield 4.6 tonnes $ha^{-1}$ (the carbon density per mass unit of oil being approximately double compared to that of starch) giving 1.4 tonnes oil $ha^{-1}$ which is comparable to oil yields of rape seed.

Increased knowledge of oil biochemistry in plants is of crucial importance for the development of novel oil crops for a sustainable plant oil production in the future. In this perspective, it is my hope that the work included in this thesis will contribute to this understanding. It is this understanding that in the future will enable us to engineer biochemical pathways in plants and redirect fixed carbons from photosynthesis into oil.

# 2 Background

## 2.1 Partitioning of carbon to different storage compounds within the seed

The seed is one of the most important biological structures in agriculture. Seeds are designed to carry all the genetic material and all the nutrients that are required to establish the next generation of the species. The stored nutrients provide the germination process with building blocks and energy until photosynthesis fuels further growth of the seedling. Atmospheric carbon is fixed through photosynthesis in the leaves and is either directly transported as free sugar to another part of the plant, or stored as starch as a transient carbon sink of the plant during day time. During night-time, starch is remobilized to support sugar synthesis, export, and respiration in different parts of the plant. Sucrose is the main form in which carbon is transported within plants (Porter, 1962) and therefore regarded as the main carbon precursor for carbon storage compounds in the developing seed.

Carbon partitioning in plants can be at many different levels, for example at whole plant level (source-sink relations, carbon translocation), organ level (for example the seed), and at tissue level (for example different tissues within the seed). In this thesis, the focus will be on the carbon partitioning between different storage compounds in different tissues within the seed.

The seed is a very strong carbon sink during seed filling when sucrose and other nutrients are channeled into different storage products within the seed. Sucrose translocation to the seed can either be symplastic (from cell to cell through the cytoplasm via plasmodesmata) or apoplastic (through the space outside the plasma membrane). Where plasmodesmata between different tissues are not continuous, sucrose transport must be apoplastic (ap Rees, 1992; Bewley & Black, 1994). When sucrose is translocated through

an apoplastic way, it can either be taken up by the cell directly as sucrose via proton-coupled transporters in the plasma membrane (Bush, 1993), or hydrolysed to hexoses by cell wall invertases that are subsequently transported by proton-coupled hexose transporters in the plasma membrane (Rausch, 1991). Thus, the form in which carbon enters the seed is both dependent on the type of transport and, in the case when it includes apoplastic transport, on the activities of the cell wall invertases and the sugar transporters in the plasma membrane. Maize is an example of a cereal that requires cleavage of sucrose by cell-wall invertases for normal seed filling (Cheng *et al.*, 1996) whereas in wheat the sucrose is thought to mainly move intact from the phloem in the crease to the endosperm (Sakri & Shannon, 1975; Ho & Gifford, 1984; Ugalde & Jenner, 1990). Sucrose unloading in wheat and barley occurs symplastically along the length of the single vascular bundle running in the crease and then pass three different cell layers before entering the endosperm cells apoplastically (Thorne, 1985).

The same tissues within a seed possess multiple biochemical capacities for the synthesis of different storage products. Typical storage products in seeds are starch, protein, and oil. However, there is a broad spectrum of variability in the partitioning of carbon within the seeds of different species. This results in seed compositions that make various species being appreciated for different economically valuable products. For example, cereals store mainly starch and protein in the seeds, soybean seeds are mostly appreciated for its high quantity and quality of the protein, whereas rape seeds are harvested for its high oil content. An interesting question is; what determines the types and proportions of the reserves laid down in the seed? Physiological differences between different types of seeds are causing some of this variation, but it is also governed by the regulation mechanisms of carbon partitioning within the seed. Also environmental parameters can affect the proportions of different storage products in a seed.

## 2.2 Physiological differences between different types of seeds

There are three main patterns of storage deposition in seeds (Harris *et al.*, 1993). In the non-endospermic dicotyledons (for example *Pisum sativum*; pea) the seed is completely filled with the embryo (Fig. 2a). The major parts of the embryo consist of modified leaves (cotyledons) in which the nutrients are synthesized and stored. This tissue remains alive in the mature seed and therefore keeps the whole metabolic machinery for converting the storage nutrients into building blocks during germination that can be used for embryo growth. In the endospermic seeds (for example the cereals) the

major nutrient reserves are present in the endosperm that is close to, but not directly connected to, the embryo (Fig. 2b). Strictly speaking, in botanical terms the dispersal unit of cereals is actually not a seed but a fruit of the type caryopsis (Bewley & Black, 1994), however, the commonly used word seed will be used throughout this thesis. The embryo of cereal seeds is usually an oil-dense tissue but typically makes out a very small part of the total seed weight and is therefore not of great importance for the total nutritional value of the seed. The endosperm and the aleurone layer (which is only a few cell layers thick) are triploid tissues originating from the fusion of one haploid pollen (sperm) nucleus with two polar nuclei in the megaspore mother cell. The endosperm of cereals undergoes programmed cell death upon maturation (Young & Gallie, 2000) and is therefore only serving as a nutrient storage of the seed and thus has no capacity for *de novo* synthesis of enzymes or metabolites in the germinating seed. Degradation of the stored nutrients in the endosperm is therefore dependent on enzymes secreted from the surrounding tissues (the aleurone and scutellum) upon germination (Bewley & Black, 1994). The scutellum, a structure originating from a reduced cotyledon in monocotyledons, has an additional function during this process to absorb nutrients released from the endosperm and then transfer them to the growing embryo (Brown & Morris, 1890; Bewley & Black, 1994). The third group of seeds, the endospermic dicotyledons (Fig 2c) store the nutrients partly in the embryo, and partly either in an endosperm as in *Arabidopsis thaliana* and *Ricinus communis* (castor bean) or in a perisperm (derived from the nucellus, a diploid maternal layer) as in *Nicotiana tabacum* (tobacco). The endosperm of dicotyledonous seeds is not

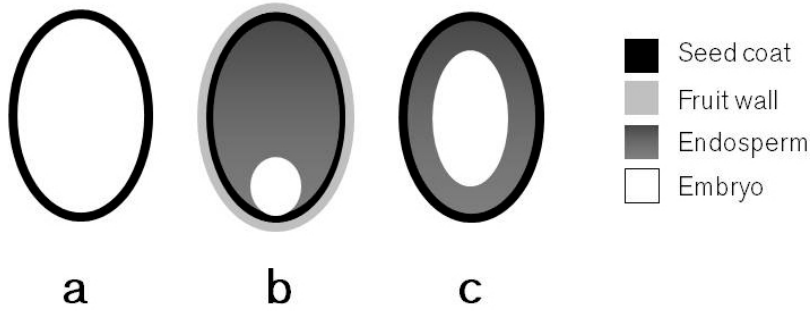



*Figure 2.* Physiological differences (tissue distribution) between the three major seed types (After Harris *et al.*, 1993). Non-endospermic dicotyledons (a, for ex. pea), endospermic monocotyledons (b, for ex. The cereals), endospermic dicotyledons (c, for ex. *Arabidopsis*). For details see the text.

going through programmed cell death and thus keeps the capacity of physiological activity during germination as reported for castor bean (Hutton & Stumpf, 1969).

## 2.3 Biosynthesis of oil and starch in the seed

The carbon deposited into the seed is the starting substrate for many different pathways of which two are starch and oil biosynthesis. As discussed above, the carbon enters the endosperm cells either as hexoses (glucose; Glc, and fructose; Frc) or as sucrose. In general, the fate of hexoses entering the cytosol is immediate phosphorylation to Glc-6-P, Frc-6-P, and Glc-1-P (ap Rees, 1992). The fate of sucrose entering the cell cytosol is determined by the only two cytosolic enzymes known to be capable of metabolizing sucrose; alkaline invertase or sucrose synthase. The invertase catalyses the physiologically irreversible cleavage of sucrose to Glc + Frc. Sucrose synthase catalyses the reversible conversion of sucrose to UDP-Glc + Frc or ADP-Glc + Frc (UDP; uridine diphosphate, ADP; adenosine diphosphate). ADP-Glc is regarded as the main substrate for starch synthesis and the formed UDP-Glc is thought to be available for starch synthesis through conversion to hexose-P (ap Rees, 1992; Emes *et al.*, 2003).

### 2.3.1 Fatty acid and oil (triacylglycerol) synthesis

The term lipid is used for a structural diverse group of molecules that have a lot of different functions in plants. For example, all cell membranes consist of polar lipids whereas the major form of storage lipid is the non-polar triacylglycerol (oil) molecule. Fatty acids (FAs) are the long carbon chains that make out the major part of lipids and give them their characteristic hydrophobic properties. FAs do not normally exist as free fatty acids (FFAs) in plant tissues but are esterified to a glycerol backbone.

The phospholipids and galactolipids have polar head groups on the glycerol backbone that allow a bilayer formation where the FA parts face the interior of the bilayer and the polar head groups face the aqueous environments (Fig. 3a). This membrane structure provides for compartmentation of biochemical reactions in the cell and is a prerequisite for all living organisms. Examples of major phospholipids in cellular membranes are phosphatidylcholine (PC, Fig. 3b), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). The chloroplast membranes are enriched in two other lipids carrying a polar galactosyl head group, namely mono- and di-galactosyldiacylglycerol (MGDG and DGDG, respectively) (Kates, 1970; Taiz & Zeiger, 2006). The

oil molecule (triacylglycerol; TAG, Fig. 3c) consists of three FAs esterified to a glycerol backbone that lacks polar head group which makes the molecule overall hydrophobic and therefore suitable for storage in form of oil droplets.

FAs are synthesized *de novo* in the plastid from acetyl-CoA. Since acetyl-CoA cannot cross the plastid membrane (Weaire & Kekwick, 1975; Roughan *et al.*, 1979), precursors for its synthesis must be generated either in the plastid or imported from the cytosol (Fig 4). The import of carbon precursors from the cytosol depends upon specific transporter proteins on the plastid membrane (for review, see Rawsthorne, 2002; Martin & Ludewig, 2007). Several possible routes for carbon precursors for FAS in plastids have been proposed; from Glc-6-P, pyruvate, phosphoenolpyruvate (PEP), malate, and free acetate that all can give rise to acetyl-CoA (Rawsthorne, 2002). The conversion of Glc-6-P to pyruvate requires a complete glycolytic pathway which is present in both the cytosol and plastids in plants (Plaxton & Podesta, 2006) which also includes the non-phtosynthesizing plastids such as those in wheat and castor bean endosperm (Simcox *et al.*, 1977; Entwistle & ap Rees, 1988). Acetyl-CoA is elongated to malonyl-CoA by the action of acetyl-CoA carboxylase (ACC) which is regarded to be the first committed step in the FA synthesis pathway. Malonyl-CoA is further converted into malonyl-ACP (ACP; acyl carrier

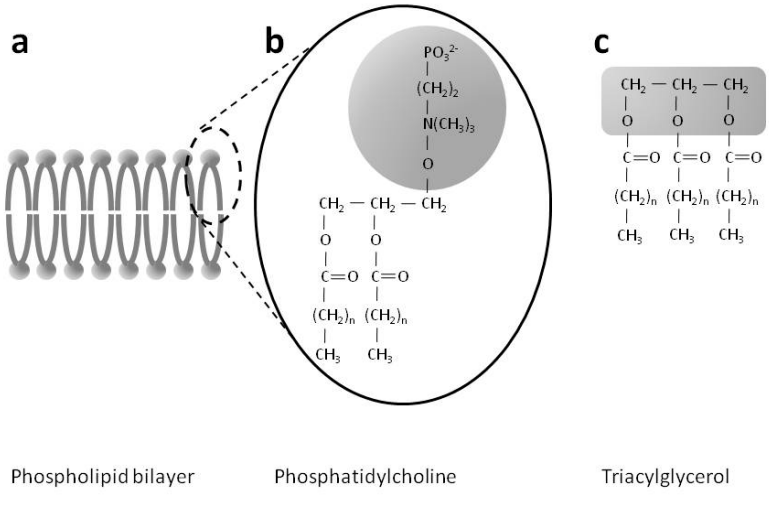
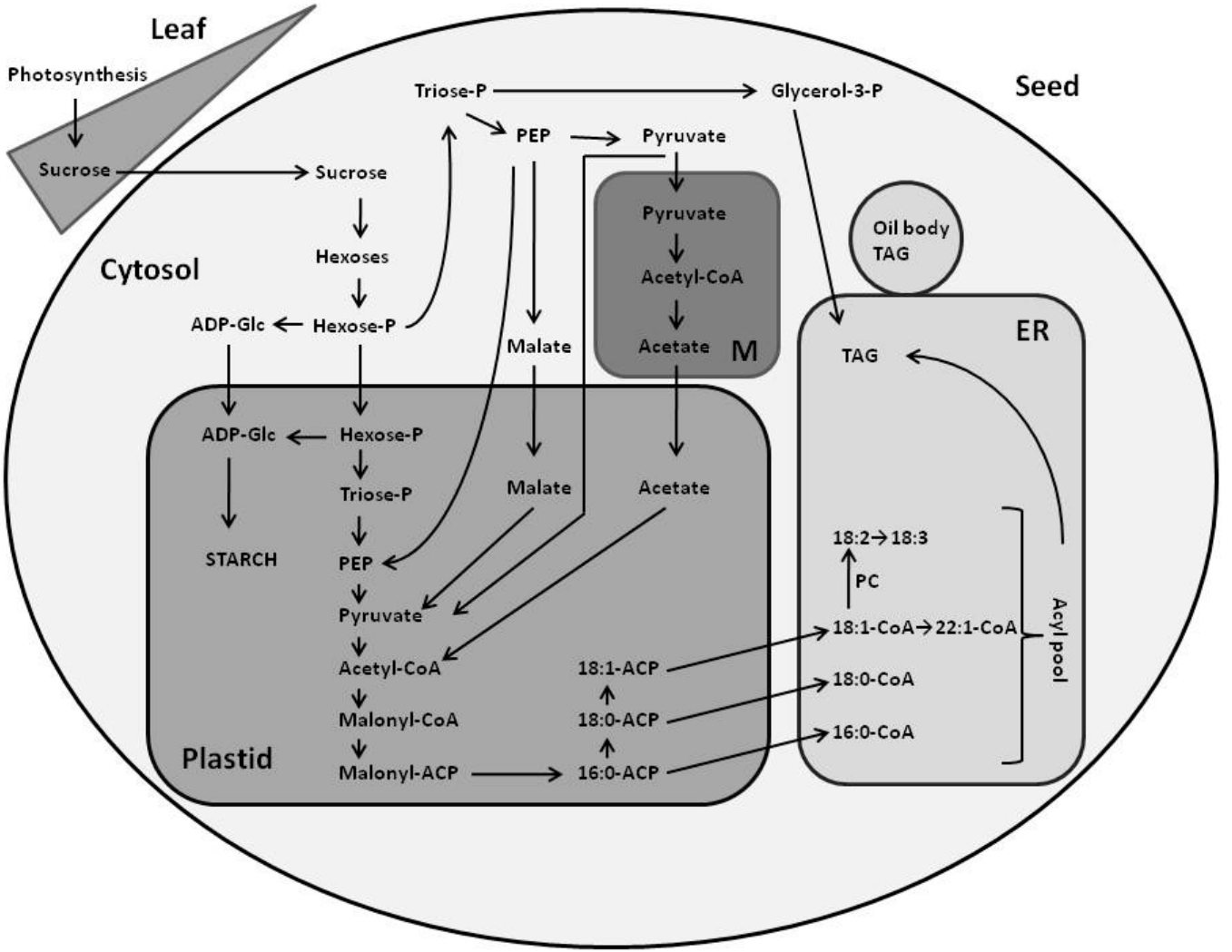



*Figure 3.* Examples of plant lipids. All cell membranes consist of bilayers of phospholipids (a). One example of a phospholipid is phosphatidylcholine (b) with a polar head group (highlighted in gray) and two acyl chains attached to the three carbons in the glycerol backbone. The major form of storage lipid in plants is the triacylglycerol (oil) molecule (c) with three acyl chains attached to the glycerol backbone (highlighted in grey).

protein) which is the substrate for the FA synthase complex, a multi-functional enzyme in plants that produces acyl chains with up to 16 carbons bound to ACP (16:0-ACP). Subsequent elongation and desaturation within the plastid give 18:0-ACP and 18:1-ACP. A thioesterase will cleave the FAs from the ACP in the plastid and the formed FFAs are channeled through the plastid membranes and exported out in the cytosol in the form of CoA esters (instead of the ACP esters that occur in the plastid). The acyl groups can then be utilized by endoplasmatic reticulum (ER) bound enzymes catalyzing for example acylation, elongation, and further desaturation. It should be noted that desaturation of 18:1 to 18:2 and 18:3 takes place while the precursor acyl group is esterified to PC (Stobart et al., 1980).

FAs from the acyl-pool can be esterified to the glycerol backbone (glycerol-3-phosphate; G3P) through different lipid intermediates in the ER and give rise to TAG that will subsequently form oil bodies. There are several enzymes involved in the transfer of acyl groups from different lipid species intermediates to give rise to TAG. However, since the aim of this PhD project is more involved in the upstream competition of carbons for starch and oil synthesis, details of these different pathways and enzymes are not covered here but can be found elsewhere (Buchanan *et al.*, 2000).

Additional to acetyl-CoA as carbon precursor, FA synthesis also requires energy (ATP) and reducing power (NADPH). The origin of carbon precursors, ATP, and NADPH for FA synthesis in plants has been shown to be different depending on both species and types of tissues. For example, in rape seeds that are green during development, carbon precursors were provided by 3-phosphoglycerate derived from Rubisco reaction and glycolysis, whereas ATP and NADPH were supplied mostly by light-driven reactions (Ruuska *et al.*, 2004; Schwender *et al.*, 2004; Goffman *et al.*, 2005; Schwender *et al.*, 2006). In soybean, which is another species having green seeds during development, the carbon precursors for plastidic acetyl-CoA were mainly supplied from triose-P (95%) with the rest originating from malate and with reducing power coming from the oxidative pentose phosphate pathway (OPPP) and pyruvate dehydrogenase (Sriram *et al.*, 2004). In non-green seeds like sunflower, carbon precursors for FA synthesis in intact embryos were mainly supplied from trios-P derived from hexoses but with a small contribution from malate, whereas OPPP was the main provider of reducing power (Alonso *et al.*, 2007). Determination of the origin of the carbon precursors for FA synthesis can be important for formulating the best strategies for engineering biochemical pathways of a plant species to enable redirection of more carbon into oil.


Leaf
Photosynthesis
Sucrose
Sucrose
Seed
Triose-P
Glycerol-3-P
PEP
Pyruvate
Pyruvate
Acetyl-CoA
Acetate
M
Oil body
TAG
Cytosol
Hexoses
ADP-Glc
Hexose-P
Malate
ER
TAG
ADP-Glc
Hexose-P
Triose-P
Malate
Acetate
STARCH
PEP
Pyruvate
18:2→18:3
PC
18:1-CoA→22:1-CoA
Acyl pool
Acetyl-CoA
18:1-ACP
18:0-CoA
Malonyl-CoA
18:0-ACP
16:0-CoA
Plastid
Malonyl-ACP
16:0-ACP

### 2.3.2 Starch synthesis

Starch is synthesized from hexose-P through the formation of ADP-Glc (Fig. 4) by the action of ADP-Glc pyrophosphorylase (AGPase). This enzyme exists in one plastidic and one cytosolic form in different tissues of plants. In the cereal endosperm, the cytosolic form is the dominating one (i.e. 85-95%), whereas there is only the plastidial form in all other non-cereal plants and in the leaves of cereals (Denyer *et al.*, 1996; Beckles *et al.*, 2001; James *et al.*, 2003). The cytosolic localization of AGPase in cereal endosperm cells can have a functional significance for partitioning of carbon into starch when sucrose amounts in the cell are high (Beckles *et al.*, 2001). In cereal endosperm, the formed ADP-Glc in the cytosol is transported into the plastid where starch is formed through the action of starch synthase and starch branching enzymes (ap Rees, 1992; Duffus, 1993), and possibly starch debranching enzymes (James *et al.*, 2003).

## 2.4 Possible sites for regulation of carbon partitioning to oil in the seed

Whether or not a given process or pathway is operating at maximum possible rate depends on several factors. A pathway can be limited by substrate availability, environmental constraints (for example to low temperature), and amount of machinery (*i.e.* enzymes). This means that even though two plant varieties are producing different amounts of a certain compound, the capacity for synthesizing that compound might not differ between the two species, but they might use their respective capacities to different extents. Substrate availability for storage compounds synthesis in a seed storage cell depends on both long and short distance transfer of carbon (*i.e.* whole plant partitioning of photosynthetically fixed carbon and transfer of sucroses or hexoses into the cell cytosol). However, carbon availability for oil synthesis within the cell depends on the competition with other pathways for the carbon. The regulation of oil synthesis inside the storage cell can therefore occur at multiple levels in the biochemical conversion of carbon into oil. To be able to increase the oil level in a high-starch

*Figure 4 (previous page).* A generalized unidirectional scheme for oil (triacylglycerol; TAG) and starch biosynthesis in the developing cereal seed (after Duffus, 1993; Bewley & Black, 1994; Rawsthorne, 2002; James *et al.*, 2003). Sucrose biosynthesis is driven by photosynthesis in the leaves. Sucrose is translocated to the seed during seed filling where the sucrose has different fates of which two are oil and starch. For details, see the text (chapter 2.3). ER; endoplasmatic reticulum, M; mitochondria, PEP; phosphoenolpyruvate, CoA; coenzyme A, ACP; acyl carrier protein, ADP; adenosinediphosphate, P; phosphate, PC; phosphatidylcholine.

producing seed like in the cereals, it might be necessary to both decrease the starch synthesizing capacity and increase the oil synthesis capacity.

Previous attempts to increase the oil content of seeds have been concentrated on seeds that already have relatively high oil content. The production of malonyl-CoA from acetyl-CoA (catalyzed by ACC) was suggested to be a potential control point for the FA synthesis pathway (Postbeittenmiller *et al.*, 1991; Post-Beittenmiller *et al.*, 1992; Roughan, 1997) and thus a potential target for genetic engineering to achieve increased oil content of seeds. However, the altered expression of ACC in seeds of *A. thaliana* and rape did not substantially affect the amount of lipids accumulated (Roesler *et al.*, 1997; Thelen & Ohlrogge, 2002a; Thelen & Ohlrogge, 2002b). Attempts to increase the oil content in rape seeds by increasing the transfer of acyl groups to the glycerol backbone during TAG assembly through the overexpression of a yeast lysophosphatidate acyltransferase (LPAT, Taylor *et al.*, 2002) and a diacylglycerol acyltransferase (DGAT, Weselake *et al.*, 2008) only gave moderate increases of lipid accumulation (up to 14%). The highest increase in lipid content of seeds reported this far was achieved by increasing the supply of G3P for TAG assembly through the over-expression of a yeast G3P dehydrogenase in rape seed, which resulted in 40% higher FA content in seeds (Vigeolas *et al.*, 2007). However, it should be noted that the oil content in untransformed seeds was extremely low (24% on dw basis) and the oil content in the best transgenic lines were far below what is normally seen in rape seeds.

Other promising targets of genetic change to increase the oil concentration of seeds more drastically are transcription factors. These are proteins that bind to DNA which can either activate or repress the transcription of a specific gene. There are several reports on transcription factors that have a strong regulatory role in the partitioning of carbon into oil in seeds with relatively high oil content. The *wri1* mutant in *A. thaliana* showed a phenotype with wrinkled seeds with 80% deficiency in oil biosynthesis compared to wild type plants due to reduced activity of several glycolytic enzymes (Focks & Benning, 1998; Baud *et al.*, 2007). It could not be distinguished from their study if the reduced glycolytic activities observed in the *wri1* mutants were preferentially localized in the cytosol or plastid. However, it is interesting to note that the activity of one of the enzymes that was strongly reduced in the *wri1* mutant, the phosphofructokinase (which phosphorylates Frc-6-P into Frc-1,6-bi-P), is exclusively present in the cytosol (Plaxton & Podesta, 2006). The *LEC1*, *LEC2*, and *FUS3* genes are all key transcriptional regulators of seed

maturation in *A. thaliana* which have shown interaction with regulators involved in oil biosynthesis (for review, see Santos-Mendoza et al., 2008). In *A. thaliana*, overexpression of *LEC1* induced elevated expression of more than half of the genes involved in FA synthesis (Mu et al., 2008), and the regulatory action of the transcription factor LEC2 on FA metabolism was shown to be dependent on WRI1 (Baud et al., 2007). Other examples of transcription factors that regulate the oil content in seeds are the *Dof4* and *Dof11* genes from soy bean that by overexpression in *A. thaliana* induced the expression of one of the subunits of ACC which resulted in up to 22% higher FA content in seeds (Wang et al., 2007). However, it should be mentioned that the effect of overexpression or downregulation of a transcription factor proved to regulate oil synthesis in one plant tissue and species might not have the same effect in another plant and might be dependent on various other factors affecting the oil synthesis capacity.

# 3 Carbon partitioning between starch and oil in oat and *Arabidopsis thaliana*

## 3.1 Aim and objectives

The overall aim of the PhD project was to study different aspects of carbon partitioning between carbohydrates and oil in the widely used model plant for oil seeds; *A. thaliana*, and in a cereal crop; oat. The specific objectives were to:

-determine if elevated atmospheric $CO_2$ induce altered carbon partitioning between oil and starch in *A. thaliana* (paper I) and in oat

-follow the deposition of oil and other storage compounds in different parts of oat seeds during development to locate temporal and spatial differences between cultivars with different total oil content (Paper II)

-follow the mobilization of oil and carbohydrate reserves in different parts of seeds during germination to find out if there are any differences in the usage of reserves in cultivars having different oil contents in the endosperms (Paper III)

-develop an *in vitro* liquid culture for oat seeds on detached panicles that allows carbon partitioning studies using radioactive substrates (Paper IV) and ODN (oligodeoxynucelotide) inhibition studies during seed development

## 3.2 Plant material

*A. thaliana* can be regarded as a model plant for high-oil producing crops such as rape (*B. napus*) even though this comparison should be considered with care due to the differences in seed characteristics between the two species (Li *et al.*, 2006). Seeds of *A. thaliana* Heynh Columbia (Col-0) were supplied from Lehle Seeds (Round Rock, TX, USA).

In all studies of oat seeds two different cultivars were used; one high-oil cv. Matilda (10% oil) and one medium-oil cv. Freja (6% oil) from Svalöf Weibull AB (Svalöv, Sweden). Cv. Matilda is originating from high-oil lines out of cv. Freja, and both cultivars share the same parental lines (Vg 75842 x Dula, personal communication A. Ceplitis, Svalöf Weibull AB, Svalöv, Sweden). Definitions of seed developmental stages can be found in Appendix A. In all experiments, only the seed in the first floret lemma of the two seeds in each spikelet (the bigger seed) was used in analyses to ensure uniform seed size.

## 3.3 The effect of elevated atmospheric $CO_2$ level on plant lipid metabolism

Plants have had to adapt to elevated atmospheric $CO_2$ throughout evolutionary history (Post et al., 1990). However, it is the rate of the present increase that is extraordinary (IPCC, 2007), and that raises questions about how this change in photosynthetic conditions will affect plants. The carbon-fixing enzyme ribulosebiphosphate carboxylase/oxygenase (Rubisco) is not saturated with $CO_2$ at current levels (appr. 380 ppm), causing the oxygenase reaction of the enzyme to compete with the carboxylase reaction, which leads to carbon loss of the plant. Elevated atmospheric $CO_2$ would therefore, at least in theory, result in a more efficient fixation of carbon in photosynthesis. The increase of atmospheric $CO_2$, that is predicted to reach approximately 800 ppm in the end of this century, together with increases in temperatures (IPCC, 2001) has been simulated both in the field and in controlled climate chambers in order to study the effects on plant growth, productivity, and nutritional quality. The expression and translation of genes involved in photosynthesis was shown to decrease in *A. thaliana* leaves when exposed to elevated $CO_2$, showing that photosynthetic reactions are affected (Robertson *et al.*, 1995; Cheng *et al.*,

1998). A range of $C_3$ and $C_4$ plant species were shown to increase total biomass production (with average increases of 40 and 20%, respectively) and increases in allocation of mass to reproductive organs can be expected under elevated $CO_2$ (Jablonski *et al.*, 2002; Poorter & Navas, 2003). It is therefore of agricultural interest to know whether an elevated $CO_2$ will alter the carbon partitioning into oil and the conditions for lipid metabolism in oil-producing crops in the future.

The effect of elevated $CO_2$ on starch and sugar metabolism in plants have been extensively studied (for reviews see Stitt, 1991; Heineke *et al.*, 1999; Sharkey *et al.*, 2004) whereas that on plant lipid metabolism were dealt with in only a few studies. Changes in oil productivity of a crop could be due to both altered seed composition and total seed yield (due to changes in individual seed mass and/or total seed mass). Lipid composition of plant leaves have been shown to be affected by environmental factors such as temperature and light (Harwood, 1994) and studies on wheat leaves showed that alterations in lipid composition can also be expected for growth at elevated $CO_2$ (Robertson & Leech, 1995; Williams & Harwood, 1997; Williams *et al.*, 1998a; Williams *et al.*, 1998b). As leaf lipids are major components of cell membranes and also involved in signaling systems in plants, their metabolism can influence the development of an organism.

### 3.3.1 The effect of elevated atmospheric $CO_2$ level on lipid composition of *A. thaliana* seeds and leaves

It is well known that elevated $CO_2$ levels induce increased accumulation of starch in plant leaves, including *A. thaliana* (Cheng *et al.*, 1998; Li, PH *et al.*, 2006; Teng *et al.*, 2006), but the effect of elevated $CO_2$ on the diurnal variations in starch concentration was not reported before in this plant. Our results showed that the leaf starch concentration was 25% higher at the end of the day in plants exposed to elevated $CO_2$ compared to ambient level (380 ppm). Moreover, starch reserves were almost depleted by the end of the night at ambient level of $CO_2$, whereas there were still a few percentages left at elevated $CO_2$.

In our study, no change in oil productivity of *A. thaliana* seeds was observed, neither due to changes in seed oil content nor total seed mass of plants exposed to elevated $CO_2$ level (800 ppm) throughout development, compared to ambient level. These results are consistent with those of the studies on the effect of elevated $CO_2$ on the reproduction mass of eight genotypes of *A. thaliana* (Ward & Strain, 1997), and on the oil content of rape seed (Frick *et al.*, 1994). Thus, the increased carbon flow through

photosynthesis at elevated $CO_2$ concentrations demonstrated by the higher amounts of starch in the leaves of *A. thaliana* did not increase the total amount of carbon transported to the sink during seed filling.

The most pronounced changes induced by elevated $CO_2$ in *A. thaliana* leaf lipids in our study could be targeted to the chloroplast. This is not surprising since an elevated $CO_2$ environment is supposed to give a more efficient photosynthesis as discussed above (see chapter 3.3). In our study, elevated $CO_2$ induced a significant decrease in the relative amount of MGDG in total leaf lipids, as well as a pronounced decrease in the ratio of 16:1*trans* to 16:0 FAs in PG. Among different cellular membranes, the photosynthesizing membranes of the chloroplasts are highly enriched in MGDG (55 % of total chloroplast lipids, Taiz & Zeiger, 2006). PG containing 16:1*trans* acyl groups is only existing in the thylakoid membranes of eukaryotic photosynthesizing organisms (Selstam, 1998) and the involvement of this lipid in stabilizing LHCII complexes and grana stack formation have been extensively studied (for review, see Trémolières & Siegenthaler, 1998). Interestingly, previous reports showed that elevated $CO_2$ induced a decreased ratio in grana to stroma thylakoids in several plant species including *A. thaliana* (Kutik *et al.*, 1995; Griffin *et al.*, 2001; Teng *et al.*, 2006). This suggests that the relative decreases in both MGDG and 16:1*trans* observed in our study is as a result of the decrease in the grana to stroma thylakoid ratio induced by elevated atmospheric $CO_2$ as discussed in Paper I. Our findings also serve as an example of when an environmental parameter influences specific lipids and FAs that are connected to defined functions in cell membranes.

### 3.3.2 The effect of elevated atmospheric $CO_2$ level on oat seed composition

Cereal crops are important food suppliers all over the world and any environmental factor that causes alteration of yield or quality can give major effects on world trade. The stimulating effects of elevated atmospheric $CO_2$ on growth, yield, and reproductive output of cereals have been reported in several previous studies, even though it was suggested that the responses of monocotyledons may be smaller than those of dicotyledons (Poorter & Navas, 2003). A few studies concerning the effect of elevated $CO_2$ on oat have been reported and showed that in general, biomass and seed yield increased but with large differences between varieties (Sæbø & Mortensen, 1996; O'Donnell & Adkins, 2001; Johannessen *et al.*, 2005). However, no study was conducted on the effect of elevated $CO_2$ on the seed lipid content in oat seeds. In our study, two different oat cultivars with different

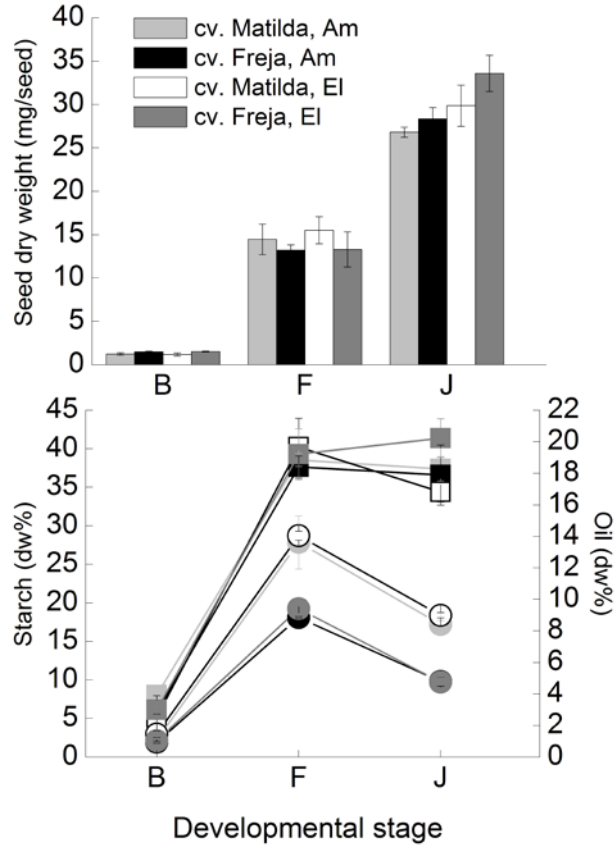



*Figure 5.* Seed weight (upper graph) and starch and oil concentration on dw basis (lower graph) of oat seeds at different developmental stages (B, F, J, see Appendix A for definitions) of cultivars Matilda and Freja grown at ambient (Am) and elevated (El) $CO_2$ levels (unpublished results). Circles; oil concentration (right axes), squares; starch concentration (left axes). Results are mean ±standard deviation.

oil content (cv. Matilda and Freja, 10% and 6% oil respectively) were grown in controlled climate chambers to investigate if elevated atmospheric $CO_2$ (800 ppm) induced any change on the partitioning of carbon into starch and oil in the kernel. Starch and oil contents were determined at three different developmental stages during kernel development (st. B, F, J, see Appendix A).

Treatment effects were analyzed by analysis of variance using the general linear model and significant mean differences were calculated using pairwise

comparison with the method of Tukey. No significant effects of elevated $CO_2$ (800ppm) was observed in seed mass or composition at early stages of development compared to at ambient levels (Fig. 5). However, in mature seeds, seed dry weight was increased by 4.2 mg/seed at elevated $CO_2$ compared to ambient level ($p \leq 0.01$). There was no significant effect of $CO_2$ level on oil concentration during any stage of seed development (Fig. 5) which is in agreement with a study of the effect of different environmental parameters on wheat seed lipid content where elevated $CO_2$ level alone were shown to have no effect (Williams *et al.*, 1994). However, there was an interaction effect between $CO_2$ level and oat cultivar for starch concentration in mature seeds in our study: At elevated $CO_2$ level, starch concentration was 7% higher in cv. Freja compared to in cv. Matilda ($p \leq 0.05$) whereas there were no significant cultivar differences at ambient $CO_2$ level (Fig. 5). This inversed relation between starch and oil in cv. Matilda and Freja was also observed under greenhouse (Banas *et al.*, 2007) and field growth conditions (Å. Grimberg, unpublished results), but not when grown in controlled climate chambers (Ekman *et al.*, 2008).

## 3.4 Oat as a model crop for oil synthesis in the cereal endosperm

Oat is unique among the cereals in storing large amount of oil in the endosperm cells of the seeds with different cultivars ranging between 3-18% of oil (Youngs *et al.*, 1977; Peterson & Wood, 1997; Frey & Holland, 1999) whereas, in other cereals like wheat and barley, this range is limited to 2-3% (Price & Parsons, 1975; Åman & Hesselman, 1984). Cereals typically store the main part of the carbon reserves in the seed in the form of starch in the endosperm, and the oil is mainly confined to the embryo. However, there are some maize varieties with enlarged embryo that increases the total oil amount of the seed (Alexander & Seif, 1963). The oil palm, which is the largest source for plant oil production in the world (FAOSTAT, 2008) is, apart from the mesocarp, also storing a substantial proportion of the carbon reserves in the form of oil in the endosperm (Oo *et al.*, 1985). Taken together, these facts show that different species partition the carbon to oil and carbohydrates in very different proportions within the same type of cells.

Higher oil content of oat seeds has been shown to be negatively correlated with starch content (Peterson & Wood, 1997). Together with the fact that the major part of the oil in oat seeds have been claimed to be localized in the endosperm (Youngs *et al.*, 1977; Price & Parsons, 1979;

Peterson & Wood, 1997), this suggests that a portion of the carbon in the endosperm cells is redirected from starch to oil synthesis in high-oil oat varieties. This suggests that oat can be used as a model crop to identify the genetic and molecular switches in the partitioning of carbon between starch and oil in the cereal endosperm. To transfer this knowledge into high-yielding cereals like maize and wheat would enhance the possibilities to engineer new crops for a sustainable oil production in the future.

A number of different studies determined total oil content and FA composition in seeds of different oat varieties (Welsh, 1995). Total oat grain oil consists of three major FAs which altogether make up more than 95% of total FA content; palmitic (16:0), oleic (18:1) and linoleic acids (18:2). There have also been several studies on lipid content and composition in different parts of oat seeds (Youngs *et al.*, 1977; Price & Parsons, 1979; Peterson & Wood, 1997). However, none of these has reported data on lipid deposition during different stages of seed development. The fact that the major part of the lipids in oat seeds is localized to the endosperm also raises interesting questions about the fate of these lipids during germination. Since the oil content in the endosperm of most other cereals is very low, studies on oil degradation during germination of cereals are few and in oat they mostly concern how oil and its degradation products causes processing difficulties in the food industry (Zhou *et al.*, 1999).

### 3.4.1 Lipid deposition in oat seeds during seed development (Paper II)

Lipid deposition and composition in different parts of oat seeds during four stages of seed development (st A, E, G, and J, see Appendix A) was characterized in two cultivars of oat having different oil content (high-oil cv. Matilda 10%, medium-oil Freja 6%). Lipid analyses using thin-layer and gas-liquid chromatography (TLC and GC, respectively) showed that the main part of oat oil (approximately 90%) was localized to the endosperm, corroborating earlier findings (Price & Parsons, 1979). It should be mentioned that the aleurone tissue was included in this fraction in lipid analyses in spite the fact that it is an oil dense tissue. However, the aleurone layer is only one to two cell layers thick and can only make out a very small proportion of total oil amount in the endosperm fraction. The higher oil content of seeds of cv. Matilda compared to cv. Freja was up to 95% due to a higher lipid content in the endosperm tissue. The rest of the difference was due to a higher neutral lipid content of the embryo+scutellum in cv. Matilda compared to Freja. An unexpected finding was that a majority (approximately 80%) of final oil amounts in the mature seed was deposited

already at mid stage of development (st E), whereas starch and protein deposition continued throughout seed development.

The main difference between the cultivars in FA composition of total seed lipids was that the high-oil cv. Matilda showed a higher 18:1/18:2 ratio compared to cv. Freja. This finding is in agreement with a previous study showing that increased oil content in oat is positively correlated to the relative amount of 18:1 (Holland *et al.*, 2001). This can most probably be explained by increased flux into *de novo* FA synthesis in the varieties with increased oil concentration at the same time as $\Delta 12$ desaturase activity is unchanged. The same effect has also been observed in FA profiles of leaf lipids induced by diurnal variations (Browse *et al.*, 1981; Ekman *et al.*, 2007). In line with this hypothesis was the decrease in 18:1/18:2 ratio during the same time as the rate of oil synthesis was much reduced (between midstage of development until maturity). However, the magnitude of these changes suggested that the decrease in 18:1/18:2 during later stages of seed development was not only due to reduced FA synthesis but could also be due to turnover of TAG in the endosperm.

The localization of lipids from analyses using chromatography was confirmed using light- and transmission electron microscopy (LM and TEM respectively). An interesting finding was that the oil was stored as discrete oil bodies in the aleurone, scutellum, and embryo, whereas the oil droplets in the endosperm fused upon maturation. Oil bodies are spherical organelles packed with TAGs surrounded by a phospholipid mono-layer embedded with proteins called oleosins (Murphy, 1993). The presence of oil body proteins is thought to prevent the oil bodies to fuse and consequently to give a larger surface area available for enzymatic attack during oil degradation (Hsieh & Huang, 2004; Siloto *et al.*, 2006). In line with this hypothesis and with our results showing that oil bodies fused in the endosperm upon germination, oil fractions of mature seeds of cv. Matilda contained much more oil body proteins per amount of oil in the embryo and scutellum compared to the endosperm (Waheeb *et al.*, 2008).

Thus, our studies established that the same endosperm cells that store starch, also accumulate oil. Since the two cultivars investigated are genetically very close in origin (see chapter 3.2) and since they showed the inversed relation to starch amounts that was also observed in other oat varities (Peterson & Wood, 1997), this suggests that these two cultivars make out an excellent model system for the partitioning of carbon into oil synthesis in the cereal seed. As discussed in chapter 2.4, there are several possible sites for key determinants of the carbon partitioning between starch and oil. For example, does the high-oil oat cultivar have a lower starch

synthesizing capacity, or does the higher oil result from a more efficient oil biosynthesis in the two competing pathways? A major quantitative trait locus explaining up to 50% of the increased oil content in crosses between three oat varieties was linked to an ACC gene (Kianian *et al.*, 1999). However, this step in FA synthesis is far downstream from the competition between starch and oil synthesis for available carbons and therefore makes ACC unlikely to be the main determining enzyme for a switch in partitioning of carbon between starch and oil in the endosperm cells.

One interesting potential point of regulation of substrate availability for FA synthesis is the formation of ADP-Glc (Fig. 4). The cytosolic AGPase in cereal endosperm accounts for the major part (85-95%) of ADP-Glc formation and the cytosolic localization has been suggested to have a functional significance for partitioning of carbon into starch in the endosperm (Beckles *et al.*, 2001). Maize mutants with defects in the small or large subunit of the cytosolic AGPase (*shrunken2* and *brittle2*) show substantially reduced starch content in the endosperm (Tsai & Nelson, 1966; Dickinson & Preiss, 1969), and the *brittle1* mutant with eliminated ADP-Glc transport capacity into the plastid also show reduced starch content and causes accumulation of ADP-Glc in the cytosol (Shannon et al., 1996).

### 3.4.2 Lipid mobilization in oat seeds during germination (Paper III)

To supply the energy stored as TAG in a seed available for embryo growth during germination, the action of lipases must first release the esterified FAs from TAG. FFAs are then degraded through the β-oxidation and glyoxylate cycles and subsequently converted into sugars that can be used as building blocks in the synthesis of different compounds needed in the seedling (Clarke *et al.*, 1983; Graham, 2008). Even though lipases have been shown to be secreted from the aleurone into the endosperm during germination of cereals (i.e. barley, Jensen & Heltved, 1982), the endosperm of cereals goes through programmed cell death upon maturation (see chapter 2.2) and can therefore not serve as a site for FFA degradation. The facts that the major part of the oil in oat seeds is stored in the endosperm and that the oil bodies in this tissue fuses upon maturation (chapter 3.4.1) therefore raises interesting questions about the fate of this oil during germination.

Our studies on oat seeds during germination showed that oil stored in the endosperm was degraded in both the high-oil cv. Matilda and medium-oil cv. Freja. However, the first oil reserve to be degraded was that stored in the embryo during the first day of germination, similar to what has been reported for wheat (Tavener & Laidman, 1972). FFAs did not accumulate

in the endosperm in amounts corresponding to the release of FFAs from TAG. In fact, the FA composition of different parts of the seed indicated that the FFAs were most probably absorbed by the scutellum. This is similar to the situation in oil palm seeds during germination where a structure with similar function, the haustorium, is absorbing the FFAs released from the oil dense endosperm (Boatman & Crombie, 1958). Previous enzymatic studies on maize and oil palm have shown that the absorptive tissue of monocot seeds is a probable site for FFA degradation (β-oxidation and glyoxylate cycle) during germination (Oaks & Beevers, 1964; Oo & Stumpf, 1983; Alang *et al.*, 1988).

The results from our study showed that even if the loss of lipids from the endosperm (mainly from TAG) was similar in amount for both cultivars during the first seven days of germination, the net loss of lipids from the whole seed was higher in cv. Freja during the same period, due to considerably higher amounts of lipids formed in the embryo+scutellum in cv. Matilda. The rate of lipid transfer from the endosperm was 28% higher due to a later start of oil degradation in cv. Matilda, as compared to in cv. Freja. In addition to much higher amounts of TAGs and FFAs in the scutellum of cv. Matilda compared to cv. Freja, this tissue also accumulated up to ten times more starch during germination.

This study shows that there are large cultivar differences in partitioning of carbon reserves not only when storage compounds are deposited in the developing seed but also when carbon reserves are transferred from the endosperm to the embryo during germination.

## 3.5 Metabolic flux studies in oat seeds using an *in vitro* liquid culture of detached oat panicles

To study the accumulation and degradation of different storage compounds in seeds during seed filling and germination gives valuable information about temporal and spatial differences in carbon partitioning between different varieties. However, this data does not give any detailed reasons about *why* different varieties of the same species show different ratios between different storage compounds or *where* the varieties differ in the biochemical pathways leading to these compounds. A prerequisite for such investigations in oat is the development of an experimental system that allows metabolic flux analysis using stable and radioactive isotope labeling. Metabolic flux is the flow of matter through the metabolic network. By feeding traceable substrates to the seed, the flux into and out from different storage compounds within the seed can be followed in more details. Such

studies can lead to a deepened understanding of the biochemical differences between oat varieties with different oil content in the endosperm.

There are several different approaches to quantify biochemical fluxes in plants (Ratcliffe & Shachar-Hill, 2006; Schwender, 2008) with the aim to determine rate limiting steps in different pathways that could be potential targets for genetic modification. In our study we measured net accumulation of $^{14}C$ in different storage compounds in oat seeds after incubation with U-$^{14}C$ sucrose at different stages during seed development. Since net accumulation is the result from synthesis minus degradation, the results from such studies cannot distinguish several possible reasons for higher oil in cv. Matilda compared to cv. Freja. Therefore, the results from our study on carbon partitioning between oil and carbohydrates using an *in vitro* liquid culture of detached oat panicles was only confirmative to previous results obtained from *in planta* studies. However, our results represent the establishment of an experimental system that can be used for more detailed analyses in the future.

### 3.5.1 Development of an *in vitro* liquid culture for oat seeds on detached panicles for use in metabolic flux studies (Paper IV)

An *in vitro* liquid culture of detached oat panicles was developed after similar systems reported for rice (Lee *et al.*, 2000) and wheat (Singh & Jenner, 1983) and optimized to mimic kernel development from anthesis to maturity *in planta*. In order to gain a more detailed understanding of oil deposition in oat, seed filling was examined at many more developmental stages than in our previous studies. Even though seed filling *in vitro* at optimal conditions only reached 60% of that obtained *in planta* at maturity, more importantly, oat seeds *in vitro* displayed comparable developmental growth rates and cultivar differences in oil deposition as those *in planta*. The *in vitro* system was therefore further used in preliminary studies of carbon partitioning between lipid and non-lipid compounds in oat seeds by incubation of detached panicles with $^{14}C$-sucrose during 48h at different developmental stages.

The cultivar differences in net accumulation of $^{14}C$ carbon in seed lipids were more or less expected. Of total $^{14}C$ accumulation in the seed at a very early stage of seed development (st C), 24% was incorporated into total lipids in cv. Matilda which was in contrast to only 10% in cv. Freja (please note corrected data in the corrigendum of Paper IV). The largest absolute amounts of $^{14}C$ incorporation into lipids during seed development occurred at an earlier stage (st D) whereas the largest incorporation of $^{14}C$ in the non-

lipid faction occurred at st E, in both cultivars. The data shows that there are both temporal and cultivar differences in regulation of the partitioning of carbon into oil and carbohydrate synthesis during oat seed development. If not taking possible turnover of lipids into account during seed development, the data suggests that the high-oil cv. Matilda has a higher flux of carbon into oil synthesis compared to cv. Freja, especially at an early stage of development. However, if this is a result of for example a higher oil synthesis capacity or higher substrate availability in cv. Matilda compared to cv. Freja cannot be distinguished from this data.

One example of a more detailed study to do in the future using the *in vitro* culture of detached oat panicles is the use of pulsed radioactive isotope labeling. A "pulse" of radioactive substrate fed into the seed during development can be traced, either during further development or during germination, to elucidate potential cultivar differences in for example turnover rates of TAG. However, these studies require that the radioactive labeled substrate specifically labels lipids only. Such a substrate could be $^{14}$C-acetate that when exogenously supplied is rapidly incorporated into FAs of plant leaves and isolated chloroplasts (Roughan *et al.*, 1978; Springer & Heise, 1989; Pollard & Ohlrogge, 1999), even though the applicability of acetate as a model substrate for FA synthesis *in vivo* has been questioned (Bao *et al.*, 2000).

### 3.5.2 Identification of key biochemical steps using pyrosequencing of genes expressed in oat endosperm

Taken together, the studies on oat seeds during development indicate that the regulation of genes involved with oil biosynthesis and/or breakdown is different in the endosperms of these cultivars and also at different developmental stages. Global gene expression studies using novel methods for ultradeep EST sequencing (Schuster, 2008) of oat endosperms is likely to identify key events regarding gene expression as well as to identify enzymatic pathways regulating the carbon flux into oil in oat endosperm. Genes expressed in oat endosperm (*i.e.* amplified cDNA libraries from mRNA) in the high-oil cv. Matilda at one early (appr. st C-D) and one late stage of development (st F-G) have therefore been sequenced using 454 pyrosequencing (Weber *et al.*, 2007; Schuster, 2008) in a collaboration project with UC Davis (Department of Plant Biology, K. Dehesh). The sequencing generated in total 700,000 fragments (average length 219 pb) that were combined to 60,000 contigs or EST's type sequences (on average approximately 350 bp long) that could be considered as representing individual expressed genes (unpublished results, personal communication D.

M. Hayden, UC Davis, USA, 2009). These sequences are now in the process of being compared to the databases housed with the National Center for Biotechnology Information (NCBI) to annotate the sequences to known functionality. The current data includes over 30,000 EST's type sequences with accurate comparisons to known genes and 30,000 sequences that are expressed in oat endosperm but that are unknown genes as of yet. It should be mentioned that the number of EST sequences of *Avena sativa* in the NCBI today is less than 8,000. Preliminary results also show that the sequence pool from the 454 sequencing display coverage of all activities of carbon metabolism in developing oat endosperm, including areas of starch and oil (unpublished results, personal communication D.M. Hayden, UCDavis, USA, 2009). The next step will be to identify differences in gene expression between the early stage (high oil synthesis) and late stage (low oil synthesis) to identify genes involved in the regulation of starch and oil synthesis.

From a total of 20,000 tag fragments sequenced from whole wheat seeds at five different stages of development using conventional sequencing methods, annotation to known gene functions showed that gene diversity was much higher at an early stage of development compared to a later stage (McIntosh *et al.*, 2007). This difference in tag representation was reflecting the cellular events with many more different metabolic and cellular events occurring at early stage compared to late stage when the dominant event is storage accumulation. If this is the case also in oat seeds, remains to be determined.

### 3.5.3 ODN inhibition in oat seeds using the *in vitro* liquid culture

ODN (oligodeoxynucleotide) inhibition is a method to transiently silence genes using short stretches of single stranded DNA that are exogenously delivered to cells. The ODN sequence is anti-sense to an expressed gene (mRNA) of interest and the hybridization forming a DNA/RNA duplex is thought to either sterically hinder the translation of the mRNA into protein or promote transcript degradation by RNase H activation. Paterson *et al.* (1977) were the first to show that exogenous single stranded DNA inhibited the translation of a complementary RNA in a cell-free system. With promise of high specificity and low toxicity, the use of antisense ODN in gene therapy was soon suggested and has since then resulted in huge amounts of therapeutic studies in animal cells (for reviews, see Rubenstein *et al.*, 2004; Opalinska *et al.*, 2006) but only a few in plant cells (Tsutsumi *et al.*, 1992; Sun *et al.*, 2005; Sun *et al.*, 2007). The method requires a carrier system that transfers the ODNs efficiently to the cells of interest, and, for

RNase H-mediated effects, subsequent efficient uptake into the nucleus. Problems with ODN uptake over the plasma membrane in animal cells have been circumvented with chemical or physical agents, or the use of liposomes as vectors, but ODN delivery still remains one of the major bottlenecks for its use in gene therapy (Opalinska *et al.*, 2006). However, in plant cells of barley, naked fluorescently labeled ODNs were shown to be taken up together with sucrose, both into leaf and endosperm cells, by feeding ODNs through detached leaves and panicles (Sun *et al.*, 2005; Sun *et al.*, 2007). Moreover, the expression of the target gene in these studies (a transcription factor involved in starch synthesis) was down regulated in both these tissues and was shown to cause altered structure of starch in the leaves. The mechanism behind the ODN uptake over the plasma membrane in plant cells in these studies was suggested to be mediated via monosaccharide transporters (MSTs) or sucrose translocators (SUTs). If the ODN inhibition technique proves to work also for other species, it would be a very powerful tool together with the use of stable isotope labeling for studying specific gene functions during transient down regulation.

As a means to determine if the antisense ODN uptake observed in barley endosperm (Sun *et al.*, 2007) was applicable for oat, single stranded DNA stretches of 17 bp with 5' amine ends (5'-GAGAGGTGGCGGTTGAG-3', antisense to a part of an oat AGPase small subunit previously cloned in our lab using degenerate primers) were fluorescently labeled using a AlexaFluor®488 oligonucleotide kit according to the protocol (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Labeled ODNs were further purified on spin columns (Illustra microspin G-25 columns, GE Healtcare, UK) to remove the non-attached fluorophore. The labeled ODNs were fed at 200 nM through detached panicles of oat in either 44 mM or 100 mM sucrose solution during 24h in the dark. Seeds were harvested (st. C-D, see Appendix A) and snap freezed in $N_2$ (l). A transverse disc (approximately 1-2 mm thick) was cut from the middle of the frozen oat seed and immediately immobilized in glycerol solution on a slide. In parallel to seed discs, small samples of fresh glumes covering the oat seeds were also mounted directly in glycerol. Confocal laser scanning microscopy was performed on a Zeiss LSM 510 Meta with settings as previously described (Sun *et al.*, 2005).

The results showed that the ODNs were taken up in both the oat endosperm (Fig. 6) and the oat glumes (Fig. 7) at both sucrose concentrations tested (only uptake at 44mM sucrose is shown here). However, future experiments will determine if the antisense ODN taken up in these tissues of oat is also having any effect on the gene expression.

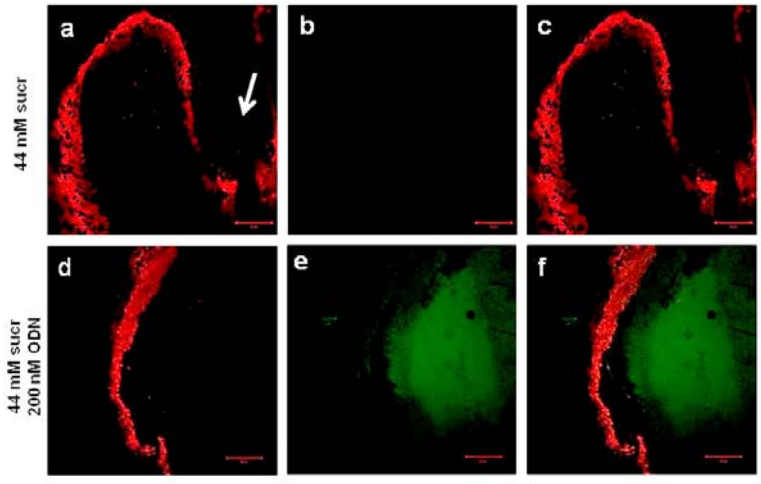



*Figure 6*. Confocal laser scanning microscopy of transverse discs from oat seeds seven days post anthesis showing oligodeoxynucleotide (ODN)-uptake in the endosperm. ODNs were fluorescently labeled with AlexaFluor488 and fed together with sucrose through the stem of detached panicles of oat during 24h. Photos a,d; autofluorescense from the seed pericarp, b,e; AlexaFluor488 label in green, c, f; fused photos. The arrow in a indicates the position of the crease of the oat grain. Scale bar 50 μm.

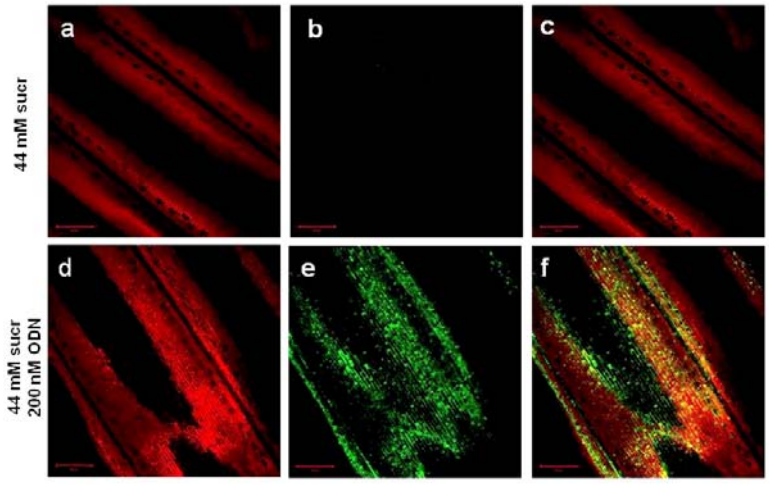



*Figure* 7. Confocal laser scanning microscopy showing oligodeoxynucleotide (ODN)-uptake in the glumes covering the oat seeds seven days post anthesis. Experiment performed as described in Fig. 5. Photos a,d; autofluorescense from the glume, b,e; AlexaFluor488 label in green, c,f; fused photos. Scale bar 50 μm.

# 4 Conclusions and future prospects

## 4.1 Conlusions

*-An elevated atmospheric $CO_2$ level did not give increased oil content in seeds of A. thaliana or oat but induced an altered leaf lipid composition in A. thaliana*
The increased carbon flow through photosynthesis at elevated atmospheric $CO_2$ concentrations demonstrated by the higher amounts of starch in the leaves of *A. thaliana* did not increase the total amount of carbon transported to the sink or to oil during seed filling. However, elevated $CO_2$ induced changes in composition of *A. thaliana* leaf lipids that are known to be part of photosynthetic membranes. Elevated $CO_2$ did not induce altered levels of oil in oat seeds.

*-Oat is suitable as a model crop for oil synthesis in the cereal endosperm*
The majority of oat seed lipids (up to 90%) were found in the endosperm cells that are also accumulating starch. Cultivar differences between a high- and medium-oil oat cultivar sharing the same parental genotypes was almost entirely due to different amount of oil in the endosperm. This suggests that the regulation of the redirection of carbons for oil synthesis differ between these oat cultivars. Oat is therefore suitable as a model plant for oil synthesis in the cereal endosperm.

*-The oil trapped in the oat endosperm is rapidly degraded during germination*
The oil reserves in the oat endosperm were mobilized in both oat cultivar bus started one day later in the high-oil cultivar compared to the medium-oil cultivar. The transport rate of lipids from the endosperm during germination was almost 30% higher in the high-oil cultivar which also accumulated higher amounts of lipids in the embryo+scutellum, compared

to the medium-oil cultivar. Free FAs released from oil in the endosperm were most probably absorbed by the scutellum.

*-An in vitro culture for seeds on detached panicles of oat was established*
An *in vitro* liquid culture of detached oat panicles was optimized to mimic seed development and cultivar differences in deposition of storage compound from anthesis to maturity *in planta*. The *in vitro* system was used in preliminary radioactive isotope labeling experiments that confirmed the cultivar differences in carbon partitioning into oil observed *in planta*.

## 4.2 Future prospects

Redirection of carbon flux from starch to oil in the cereal seed can create new high-yielding oil crops as a sustainable alternative to fossil oil in the future. Using oat as a model system, we can enhance our understanding of carbon partitioning from starch to oil in a cereal endosperm. The sequence database from the 454 pyrosequencing of expressed genes in oat endosperm will provide a large resource for future studies and allow the identification of pathways and transcription factors regulating the carbon flux into oil in the oat endosperm. In combination with metabolic flux analysis using radioactive and stable isotope labeling and potentially transient gene silencing through antisense oligodeoxynucleotide (ODN) inhibition using the *in vitro* culture of oat seeds, this will contribute to the understanding of the factors which regulate the accumulation of storage products in the cereal endosperm. This understanding can enable us to engineer enhanced pathways into plants that have the potential to redirect fixed carbon from photosynthesis into oil.

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