Micro-domains as site for storage lipid biosynthesis in seeds and algae

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Abbreviations

A_{650}	Absorbance at wavelength 650 nm of light
bp	base pairs
C	Cytosine
CTAB	Cetyltrimethylammonium bromide
cDNA	Complementary DNA
d	Day
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
DW	Dry weight
EDTA	Ethylenediaminetetra acetic acid
ER	Endoplasmic reticulum
et al.	*et alii*
FA	Fatty acid
FID	Flame ionization detector
G	Gramm
GC	Gas chromatograph
h	Hours
H_2O	Water
l	Liters
LB	Luria bertani
m	Meters
M	Mol
MetOH	Methanol
min	Minutes
OD_{600}	Optical density at 600 nm wavelength of light
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Reverse transcriptase
s	Seconds
SDS	Sodium dodecyl sulphate
T	Thymine
TAG	Triacylglycerols
TLC	Thin layer chromatography
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
URA 3	Selection marker for Uracil auxotrophy
v/v	Volume to Volume ratio
w/v	Weight to volume ratio
x g	Gravitation

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1 Introduction

1.1 Increasing need for triacylglycerol

Vegetable oils present a renewable source of energy/carbon and are becoming increasingly important to our economy (Lu et al. 2011). The majority of plant oil production comes from only 4 sources: soybean, rapeseed, sunflower and palm (Thelen and Ohlrogge 2002). The oils consisting of triacylglycerols (TAGs) are being used for a wide variety of applications. These applications comprise feed, food and feedstock for the chemical industry including biodiesel ((Durrett et al. 2008); (Lu et al. 2011)). Biodiesel resembles petroleum based diesel fuel in combustion speed, energy content, viscosity and phase changes. In addition to that biodiesel is almost sulfur free and combustion causes compared to conventional diesel fuel less carbon monoxide and fewer particulates and hydrocarbons (Knothe and Steidley 2005; Muniyappa et al. 1996).

The increasing demand for vegetable oils is gaining more and more importance in our economy. TAG world production has dramatically increased over the last decade and exceeds 170 million metric tons per year (http://apps.fas.usda.gov/psdonline/psdHome.aspx). At the same time the strong demand leads to an increase of prices. For example have the prices of palm, soybean and rapeseed oil doubled since 2000, peaking at nearly threefold higher levels in 2008 (Lu et al. 2011). Additionally the demand for vegetable oils of certain compositions and qualities to fulfill specific functions increased (Napier 2007; Napier and Graham 2010; Wu et al. 2005a). Calculation of the Food and Agriculture Organisation (FAO) estimate that until 2050 an increase of 70 % in food production is needed to meet the growing population (FAO 2010). To meet this increase in demand, higher production and modifications in quality of vegetable oil is needed. Those different needs can basically be met by two different approaches. These approaches are either conventional breeding or genetic engineering.

Conventional breeding consists of techniques partially used for thousands of years, beginning with domestication of plants starting 10.000 years ago and in several regions of the earth (Gepts 2002).

The techniques used in conventional breeding can be divided into asexual techniques like planting or grafting, or sexual techniques inbreeding (self-pollination), or outbreeding (crosspollination) (Borlaug 1983).

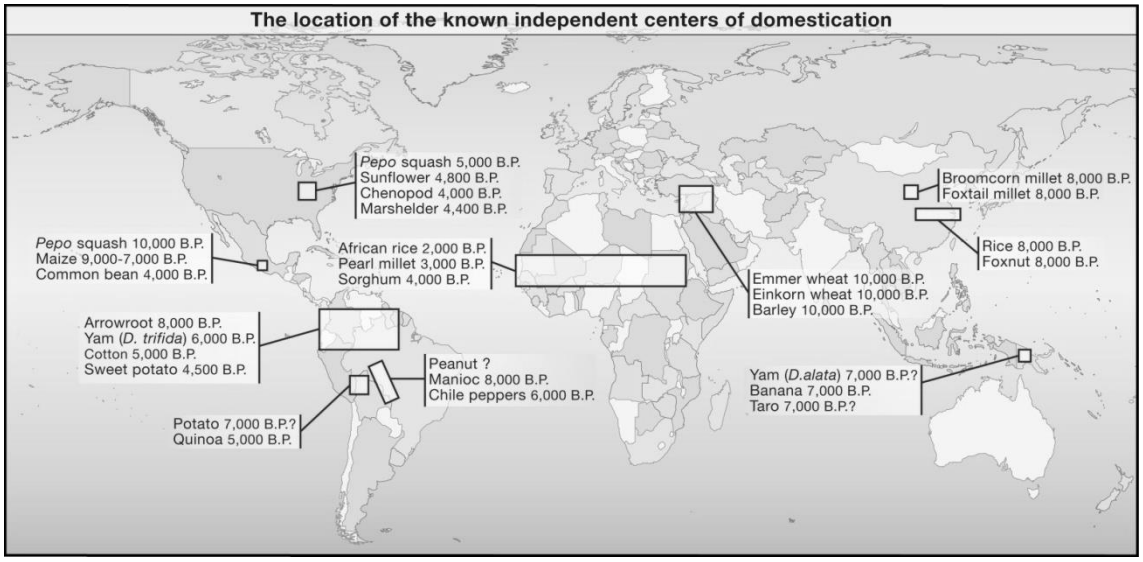

Figure 1-1: Centers of domestication.
Shown are the regions in which the indicated crops were domesticated. Basing on the currently archaeological evidence the dates for these events are given (Doebley et al. 2006).

Breeding bases on the principal that after sexual exchange of genes by cross-fertilization a selection for desired traits can be performed. Evidence suggests that natural occurring interspecific transfer by hybridization of different species or genera yielded some of our modern crop species like rapeseed, tobacco and wheat (Goodman et al. 1987). To meet different requirements in certain plant products, breeders have for a long time selected for characteristic traits. Many valuable genetic variants with alterations in the composition of fatty acids have been discovered by plant breeders. The identification of these traits was possible through screening for induced or natural variations.(Somerville and Browse 1991).

However, classical breeding bases on the occurrence of natural mutations and domesticated crops rather arise from a loss of function than a gain of function. (Gepts 2002). Naturally occurring traits can usually be attributed to recessive alleles at one not exceeding two or three loci (Ladizinsky 1985). Often the desired improvements cannot be achieved due to the not sufficient genetic diversity (Goodman et al. 1987). Furthermore is classical breeding heavily dependent on spontaneous events that occur in a very low rate. The induction of mutations by for example radiation or chemicals does increase the number of mutations (Brock 1971). However, the induced mutagenesis is

still an undirected method. In addition to that, induction of mutations influences viability and fertility of the organism (Brunner 1995).

The other way to increase TAG production may be the use of genetic/metabolic engineering. In contrast to breeding genetic engineering introduces genes into a genome by non-sexual means. That means after the fertilization of the ovule by a sperm cell (Gepts 2002). Widely used techniques in genetic engineering are for example transformation mediated by *Agrobacterium tumefaciens* or biolistics (Gelvin 2003; Sanford 1990). Genetic engineering in the last 2 decades reached a commercial stage, and several cultivars of crops that carry resistances to different pests or herbicides are been grown (Gepts 2002). An additional aim of genetic engineering is to alter vegetable oils in a way that they contain fatty acids with greater nutritional value and for non-food and industrial applications (Lu et al. 2011).

In general three different strategies to increase the TAG yield through genetic engineering are possible: 1) increase in major crop traits, 2) channeling fluxes in favor of TAG instead of sucrose or starch, 3) TAG accumulation in vegetative tissues such as leaves (Carlsson et al. 2011).

Regarding the increase in TAG three different approaches exemplified by the buzzwords “push”, “pull” and “protect” are being followed. Either fatty acid biosynthesis is up-regulated (push), or TAG assembly is increased (pull), or the breakdown is reduced (protect). For this either heterologous proteins are expressed or the expression levels of different homologous proteins are increased or reduced (Vanhercke et al. 2013a; Vanhercke et al. 2013b). Most studies up to date followed the “pull” approach by over-expression of acyl-CoA:diacylglycerol acyltransferases (DGATs) (see 1.5) which catalyze the final step of TAG synthesis (see 1.2.1) (Andrianov et al. 2010; Jako et al. 2001; Lardizabal et al. 2008; Vanhercke et al. 2013a; Vanhercke et al. 2013b).

In the recent years it has been proposed to increase the lipid content of vegetative tissues of high biomass crops (Andrianov et al. 2010; Wu et al. 2013). However, all these attempts base on the use of arable land. Therefore a conflict between the production for food and feed might become a serious problem (Chakravorty et al. 2009). Major increase in the area of arable land cannot be expected in the coming years. This would require clearing of forest area reducing the availability of wood another feedstock of our economy (Zhang et al. 2006).

Therefore algae have been discussed to be a promising feedstock for TAG production. Consequently attempts have been made to find algae that are

capable of producing TAGs in reasonable amounts (Benemann et al. 1982) (Sheehan et al. 1998). For example was from 1978 to 1996 a program funded by the U.S. Department of Energy's Office of Fuels Development to find a renewable source of transportation fuel (Sheehan et al. 1998). This and other studies found that algae are not only capable of producing TAG but also contain fatty acids like long chain poly unsaturated fatty acids (LC-PUFA) that are of high interest due to their function in human nutrition (see 1.2) (Alonso et al. 1998) (Yongmanitchai and Ward 1991b) (Crawford et al. 1997; Eizadora et al. 2009) (Spector 1999).

1.2 Triacylglycerol (TAG)

Triacylglycerols are a storage form of energy and carbon that is conserved across kingdoms. In plants it can be found in seeds and pollen of angiosperms, seeds of gymnosperms, the spores of ferns and in unicellular algae (diatoms) (Huang 1992). TAG is interesting for the economy as it can be used for different applications like food, feed and biodiesel (Durrett et al. 2008; Lang et al. 2011; Lu et al. 2011).

The molecule itself consists of a glycerol backbone to that three fatty acids are bound by ester bounds (Figure 1-2 A). The three different positions are named sn-1 to sn-3 to identify the stereospecific position at which a fatty acid is associated with the glycerol back bone (Durrett et al. 2008). The FA usually consist of carbon chains with a length of 12 to 22 carbons (Broun et al. 1999).In most plants the saturated FAs are preferably distributed between the sn-1 and the sn-3 position. Linoleic acid preferably can be found in the sn-2 position. FAs with chain lengths over C20 are mainly found in the sn-3 position (Lawson and Hughes 1988).

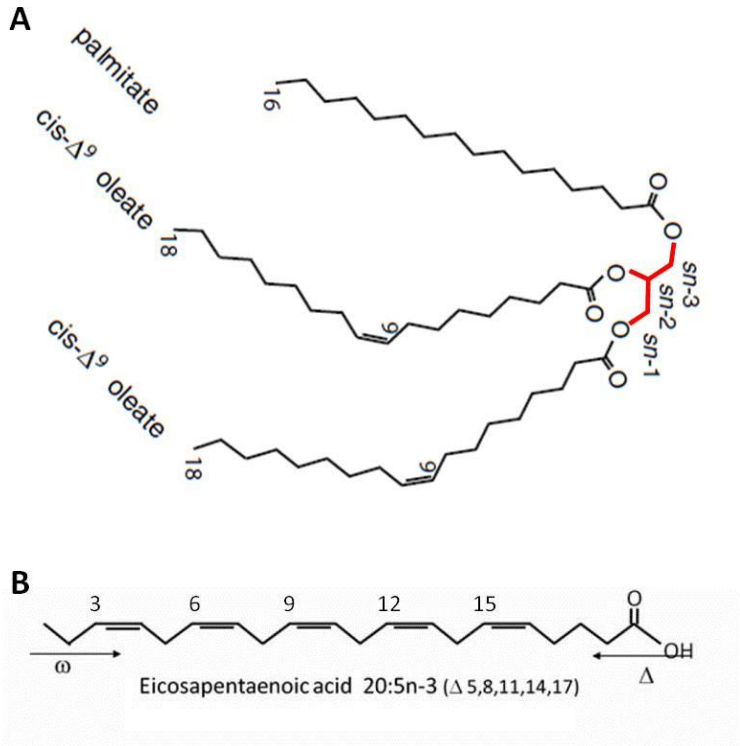

Figure 1-2: TAG molecule.
A) Example of a TAG molecule. The carbon skeleton of the glycerol backbone is indicated in red. sn-1, sn-2 and sn-3 indicate the stereospecific positions to which FA are linked by ester bounds. The molecule may possess up to three different FA moieties with different chain lengths and degrees of saturation. (B) The long chain polyunsaturated fatty acid (LC-PUFA) eicosapentaenoic acid (20:5n-3). The numbers indicate the position of the double bonds starting at the methyl end (ω-designation).

After conversion by transesterification with methanol to fatty acid methyl esters (FAMEs) TAGs may be used as biodiesel (Durrett et al. 2008).

TAGs used for human diet mainly contain five FAs. These are palmitic acid (16:0) and stearic acid (18:0) which are both unsaturated, oleic acid (18:1n-9) a monosaturated FA and linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) that are both polyunsaturated (Singh et al. 2005).

Arabidopsis thaliana (Arabidopsis) is a model plant in molecular genetics (Katavic et al. 1995). In Arabidopsis the major accumulation of lipids occurs in a relatively short and well-defined period during the middle to late embryogenesis (Mansfiled and Briarty 1996). The FAs of Arabidopsis TAG consist of carbon chains of 16 to 26 carbons length. Most FAs are monoun- or polyunsaturated (PUFAs)(Kunst et al. 1992).

Due to their function in human nutrition TAGs containing high amounts of very long chain polyunsaturated fatty acids (VLC-PUFAs) such as eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) are of special interest (Crawford et al. 1997) (Spector 1999). The nomenclature of PUFAs either follows the Δ-designation where the numbering of double bounds starts at the carboxyl-group, or the n-designation starting at the methyl end (Figure 1-2 B) (Wu et al. 2005a).

However, higher plants are capable of producing PUFAs with a chain length of 18 carbons like 18:2n-6, 18:3n-3 and even 18:3n-6. However, for the production of VLC-PUFAs the ability for further elongation and desaturations is necessary. In human diet the main sources of 20:5n-3 and 22:6n-3 is the consumption of marine fish and seafood (Hibbeln et al. 2006). Due to over-fishing of the oceans the security of this source might be at risk in future (Pauly et al. 2002). Therefore in recent years affords have been made to establish or increase the production of VLC-PUFAs in plants (Beaudoin et al. 2014; Cheng et al. 2010; Wu et al. 2005b).

However, different algae have the ability to produce VLC-PUFAs as they are the primary producers of these FAs (Alonso et al. 1998; Yongmanitchai and Ward 1991a). An example for an organism that is capable of producing EPA and DHA and storing it in TAG is the diatom *Phaeodactylum tricornutum*. Even the TAG of *P. tricornutum* contains 20:5n-3 and even 22:6n-3 (Alonso et al. 1998; Yongmanitchai and Ward 1991a) (Eizadora et al. 2009). Depending on the strain and the growth conditions 20:5n-3 accounts for up to 35 % of its total fatty acid content (Yongmanitchai and Ward 1992).

1.2.1 TAG synthesis

In plants and therefore in the model organism Arabidopsis TAGs are synthesized in the ER probably with the involvement of reactions at the LDs (Huang 1992). The start of the TAG synthesis in plants is the *de novo* production of FA in the plastid (Ohlrogge et al. 1979). The primarily formed FAs are palmitic acid (16:0) and oleic acid (18:1). They may leave the plastid or enter the so-called prokaryotic pathway. In the cytosol they are activated by binding to Coenzyme-A (CoA) (Johnston et al. 1997). The acyl-CoAs enter the ER either by the Kennedy pathway or by acyl-editing. The first option is called eukaryotic pathway (Roughan and Slack 1982).

Acyl chains may be further modified in the ER membrane by elongation or desaturation. Then lipids are either channeled back into the plastid to form

glycolipids or stay in the ER. In the ER the modified acyl chains either are used to form phospholipids, or stored in the form of TAGs. The direct TAG synthesis in the ER consists of only a few sequential steps starting with the Kennedy pathway (Figure 1-3). This pathway begins with the acylation of acyl-CoA:glycerol-3-phosphate (G3P) by glycerol-3-phosphate acyltransferase (GPAT) at the sn-1 position (Zheng et al. 2003). However, it is still unknown which of the eight members of the GPAT family catalyzes this reaction in Arabidopsis. The formed lysophosphatidic acid (LPA) is further acylated by the addition of a second acyl-CoA. This yields phosphatidic acid (PA) and is catalyzed by acyl-CoA:2-lysophosphatidic acid acyltransferase (LPAAT). Like for the GPAT it is to date not known which of the five Arabidopsis LPAATs actually is responsible for this specific reaction (Kim and Huang 2004).

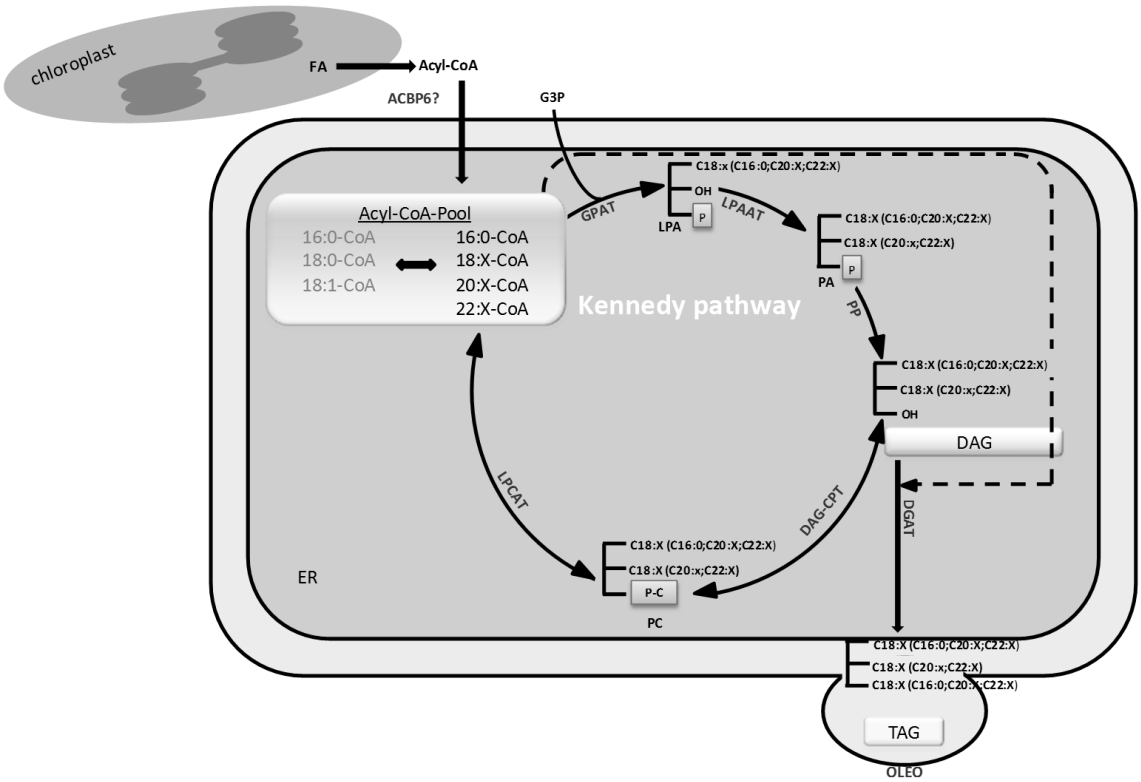

Figure 1-3: Simplified model of TAG biosynthesis in plants.
After synthesis in the chloroplast fatty acids (FA)s are being transported into the cytosol and esterified to Coenzyme A (CoA). The acyl-CoAs are channeled by an unclear mechanism, putatively by acyl-CoA binding protein 6 ACBP6) into the endoplasmic reticulum (ER). Here, they are either modified or directly enter the *Kennedy pathway.* By subsequential transfers of the acyl-groups to acyl-CoA:glycerol-3-phosphate (G3P) by glycerol-3-phosphate acyltransferase (GPAT) and acyl-CoA:2-lysophosphatidic acid acyltransferase (LPAAT) first lysophosphatidic acid (LPA) and following phosphatidic acid (PA) are formed. Dephosphorylation by phosphatidate phosphatase (PP) yields diacylglycerol, that is the precursor of either phosphatidyl cholin (PC) and triacylglycerol (TAG). The enzymes catalyzing these

reactions are diacylglycerol cholinephosphotransferase (DAG-CPT) and acyl-CoA:diacylglycerol acyltransferase (DGAT) respectively. The TAG is stored in lipid droplets (LD) that bud off from the ER. The surface of these LDs is covered with oleosins (OLEO) (modified from (Li-Beisson et al. 2013; Petrie et al. 2012)).

PA is converted to diacylglycerol (DAG) by a dephosphorylation. This reaction is mediated by the phosphatidate phosphatase (PP). Eukaryotic systems posses two classes of PPs. These are either Mg^{2+}-dependent (PP1) and play a role in synthesis of lipids, or Mg^{2+}-independent (PP2) and are involved in lipid signaling (Carman and Han 2006). In heterologous expressions in yeast the two PP1 homologs of Arabidopsis; AtPAH1 and AtPAH2 both show Mg^{2+}-dependency. Single knock-out mutants of AtPAH1 and AtPAH2 however only show slight reductions in TAG content (Eastmond et al. 2010).

Another important aspect of DAG is, that a phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) is able to transfer the phosphocholine head group of PC to DAG. This causes the incorporation of more desaturated fatty acids into DAG (Lu et al. 2009).

The by these different ways obtained DAG is subsequently either used to form membrane or storage lipids. The final step from DAG to TAG can be performed in three different ways. These ways have in common that DAG is acylated. Different however, is the donor of the acyl-chain. The most obvious way is the final step of the *Kennedy pathway* namely the acylation of the sn-3 position using a further acyl-CoA. This reaction is performed by acyl-CoA:diacylglycerol acyltransferases (DGATs) (see 1.5). The second way is the use of phosphatidylcholine (PC) as acyl donor. The enzyme phospholipid:diacylglycerol acyltransferase (PDAT) catalyzes this reaction (Dahlqvist et al. 2000). The third possible way for TAG formation is the catalysis by a diacylglycerol:diacylglycerol transacylase as it has been described for safflower seeds (Stobart et al. 1997). For Arabidopsis however this enzyme catalyzing this reaction remains to be identified.

The on this ways synthesized TAG is stored in distinct structures or storage organelles which are called oil bodies or lipid droplets (LDs) (see 1.3). It has been suggested that the final step of the TAG synthesis and the packing of the LDs localizes to distinct micro-domains of the ER (Cahoon et al. 2007; Shockey et al. 2006).

The TAG synthesis in baker's yeast *Saccharomyces cerevisiae* as well is located at the ER. The origin of acyl-CoAs of course differs from plants as yeast does not contain chloroplasts. In yeast *de novo* synthesis of FAs occurs in

the cytosol and is catalyzed by acyl-CoA carboxylase and the FA synthase complex (Hasslacher et al. 1993). *S. cerevisiae* only contains three major FAs 16:1, oleic acid 18:1 and palmitic acid 16:0 (Suutari et al. 1990). The fastest route in yeast yielding TAG is as well the acylation of DAG from the *Kennedy pathway*. The precursor in yeast also is G-3-P, which is subsequently acylated. Acylation of the sn-1 position is catalyzed by the GPAT GAT2 (Bratschi et al. 2009). The Formed LPA is acylated at the sn-2 position by the 1-acyl-G-3-P acyltransferase (AGAT) SLC1 and yields PA (Athenstaedt and Daum 1997). DAG is produced by the PP1 PAH1 through the dephosphorylation of PA (Han et al. 2007). The final step of TAG synthesis, the acylation of DAG is catalyzed by the DGAT2 type (see 1.5) DGA1 (Sorger and Daum 2002). In addition to that the two sterolester synthases ARE1 and ARE2 exhibit considerable DGAT activity (Oelkers et al. 2002a; Sandager et al. 2002). As in plants an alternative route to form TAG is via the PDAT LRO1 that utilizes a PC as acyl donor (Oelkers et al. 2002a).

In algae a TAG pathway additional to the ER-localized pathway may exist. The TAG synthesized by *Chlamydomonas reinhardtii* contains at the sn-2 position predominantly C16 FAs. DAG with these FAs is characteristic for the assembly of glycolipids by chloroplastidic acyltransferases. Additionally are the TAGs not only stored in LDs in the cytosol but also in the chloroplast (Fan et al. 2011). These LDs can even be visualized in electron micrographs of starch-less strains of this alga. Based on this Goodson and co-workers propose for *C. reinhardtii* a interaction between ER and chloroplast via LDs (Figure 1-4) (Goodson et al. 2011). In senescent leaves of Arabidopsis also TAG with typical plastidic lipids could be observed. Additionally AtDGAT1 was found to be associated with the membrane of chloroplasts, which coincided with the increased TAG content enriched in FA typical for thylakoid galactolipids (Kaup et al. 2002).

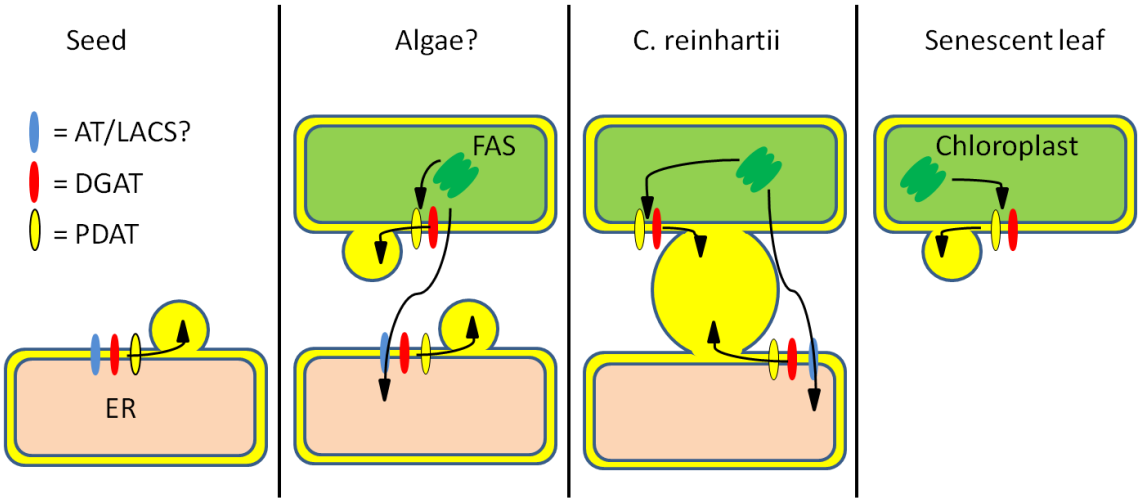

Figure 1-4: Different origins TAG.
After fatty acid synthesis (FAS) in the chloroplast fatty acids (FAs) are transported into the endoplasmic reticulum (ER) putatively by an acyl transferase (AT) or long chain acyl-CoA synthase (LACS). Triacylglycerols (TAGs) in seeds originate from synthesis by diacylglycerol acyl transferase (DGAT) or phospholipid:diacylglycerol acyltransferase (PDAT) in the ER. On the other hand are TAGs in senescent leaves produced at the chloroplast to sequester FAs that otherwise would be lost. In Algae like *Chlamydomonas reinhardtii* a combination or cooperation of plastid and ER derived TAG synthesis have been proposed.

1.3 Lipid droplets (LDs)

After synthesis TAGs need to be stored. As it is a highly hydrophobic substance it cannot simply be transported into the aqueous cytosol. Therefore TAG is stored in specific cytosolic organelles called lipid droplets (LDs) (Laibach et al. 2014). Depending on the organism LDs have a diameter between 0.2 and 2.5 µm (Huang 1992). The LDs in seeds have the function of long-term storage of TAG (Murphy et al. 2001). LDs consist of a hydrophobic core surrounded by a monolayer of phospholipids in which proteins are embedded. The lipophilic acyl chains of the phospholipids are oriented towards the TAG core, the hydrophilic head groups interact with the surrounding cytosol (Huang 1992; Yatsu and Jacks 1972). LDs contain besides TAG and smaller amounts of DAG and free FAs (Huang 1992). Cytoplasmic LDs in plants are, depending on the developmental stage present in nearly all cell types and are not confined to storage tissues (Murphy et al. 2001).